The Journal of LABORATORY and CLINICAL MEDICINE

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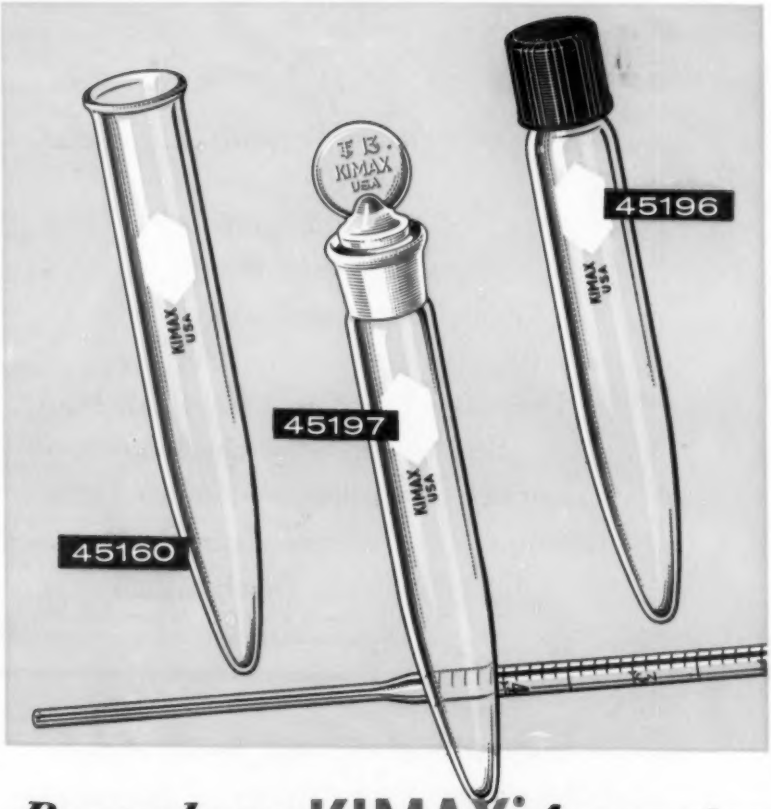

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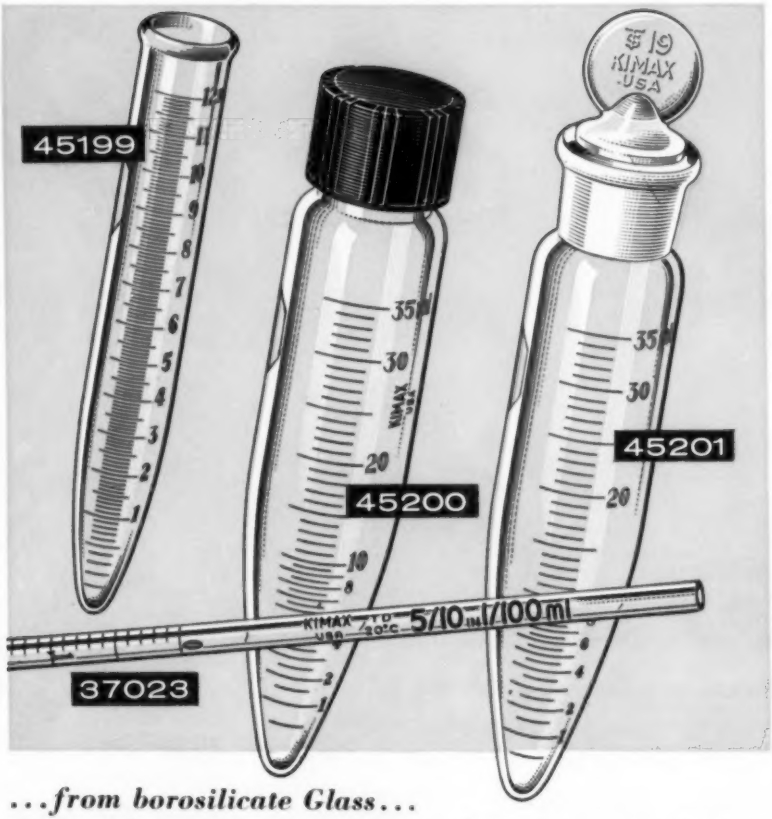

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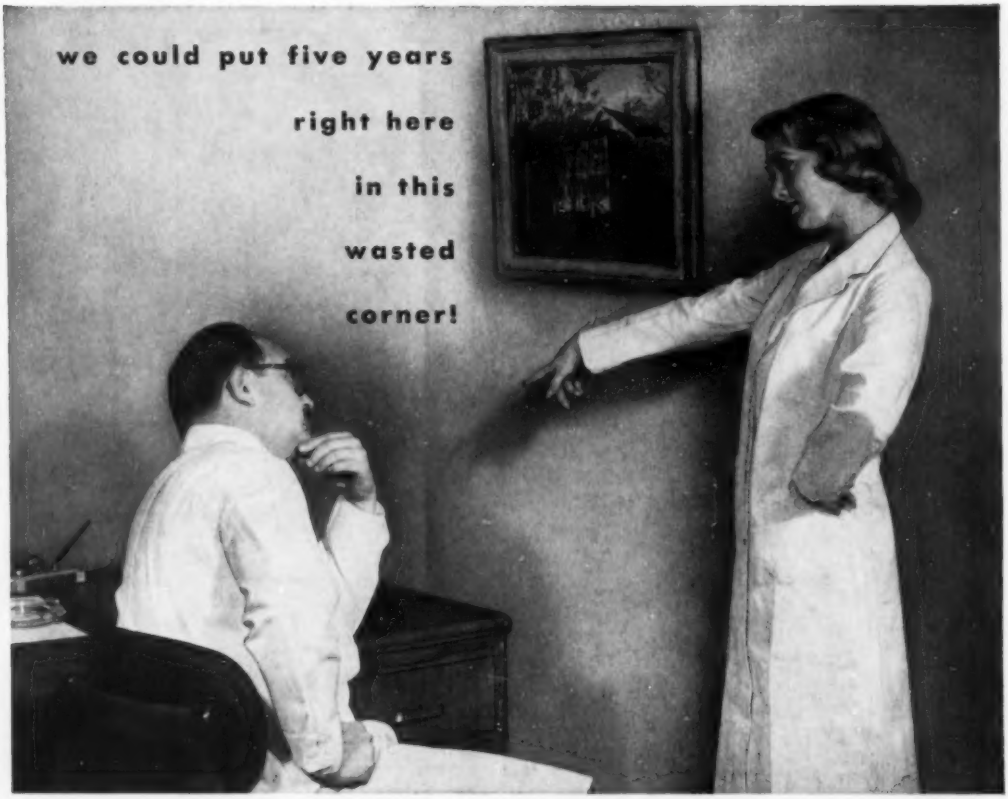

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* Peterson, E. T., Walford, R. L., Figueroa, W. G. and Chisholm, R. Ox Cell Hemolysins in Infectious Mononucleosis and in Other Diseases. Am. J. Med. *21(2):* 193-199 (Aug.) 1956. (Hyland Laboratories will send you a reprint of this article promptly, at your request.)

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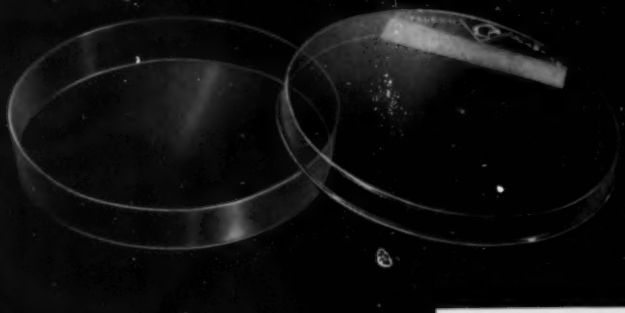

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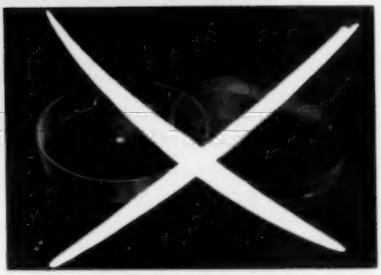

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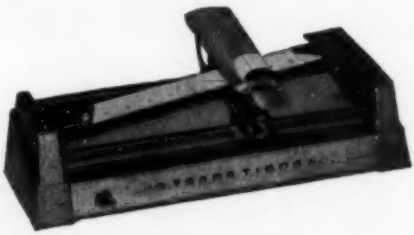

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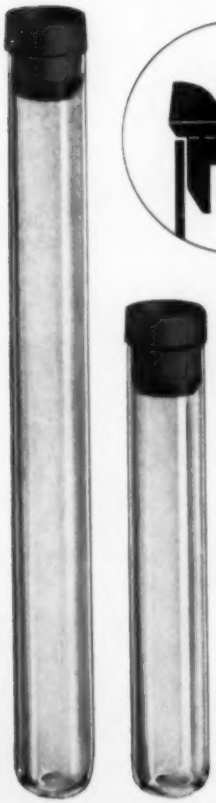

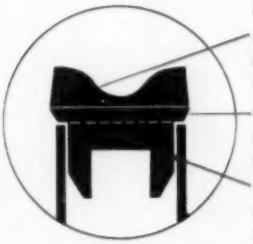

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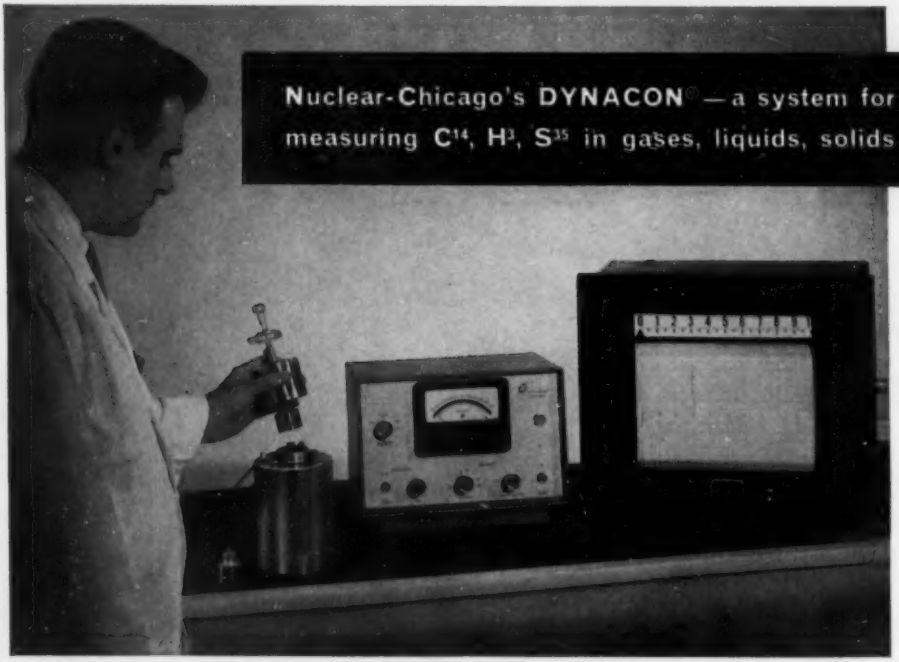

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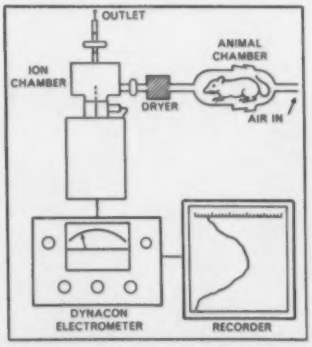

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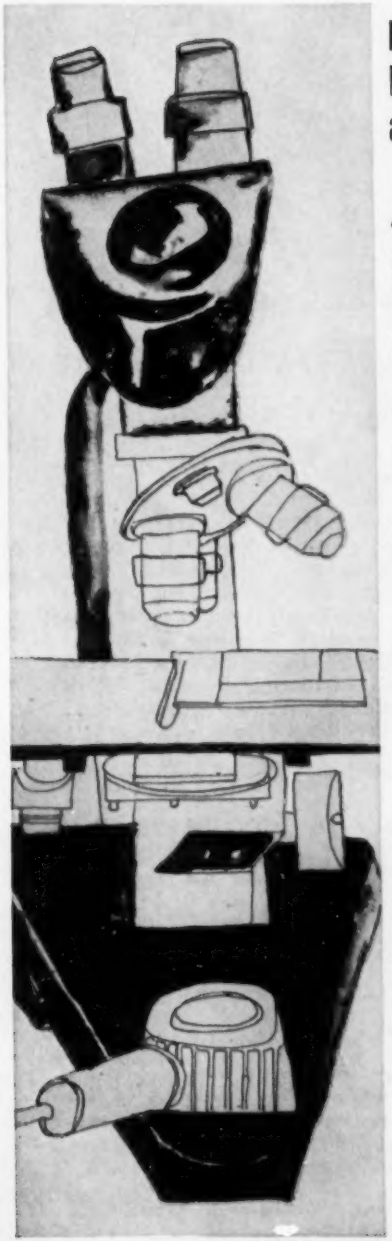

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This is what the physician tells the patient:
Start test immediately on arising, without eating or drinking anything for breakfast.

START

Urinate.
Do not keep this urine.

Tear open the small packet and swallow the 2 tablets with a glass of water.

1 HR. LATER

Urinate. Save urine in jar marked "control urine".

Open large packet. Pour contents into ¼ glass of water.

Stir well and drink it. (The granules do not dissolve.) If granules remain, add a little more water and drink them down.

2 HRS. LATER

Urinate. Save urine in jar marked "test urine".

Test Procedure

Each box of DIAGNEX BLUE has a color comparator block with two color standards—one representing color intensity of 0.6 mg. azure A, and the other 0.3 mg. azure A. Color comparison should be made against a suitable light source.

A. 1. Dilute the control and test urines with water to 300 cc. each.

2. Fill two test tubes with approximately 10 cc. of control urine each, and fill a third test tube with about 10 cc. of the test urine.

3. Place the test urine tube in the middle slot of the comparator and the control urine tubes in front of the two color standards.

4. If the color intensity of the test urine is equal to or exceeds that of the 0.6 mg. standard, the patient has secreted free gastric hydrochloric acid and the test is complete.

B. 1. If the test sample color is less intense in color than the 0.6 mg. standard, acidify all samples with 2 drops of diluted (10%) hydrochloric acid. Heat the three test tubes in a boiling bath for 10 minutes. (Boiling may decolor sample, but color will reappear on cooling.) Remove tubes from the bath and allow to cool for 2 hours. Compare color intensity as in A3 and A4.

2. When the color of the test specimen falls between the 0.6 mg. and the 0.3 mg. standards, this is presumptive evidence of hypochlorhydria. When the color of the test specimen is *less* intense than that of the 0.3 mg. standard, this is presumptive evidence of achlorhydria.

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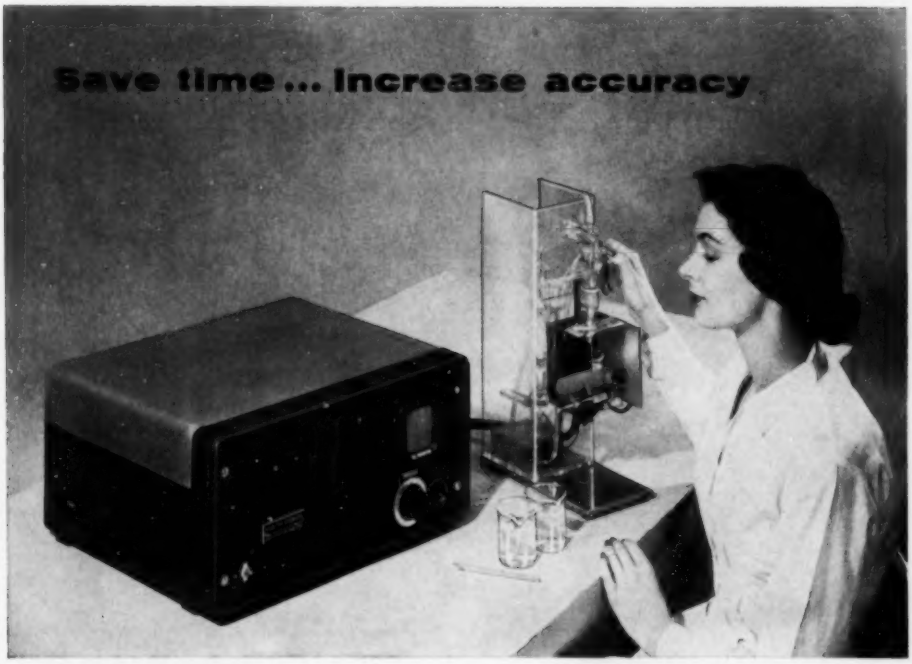

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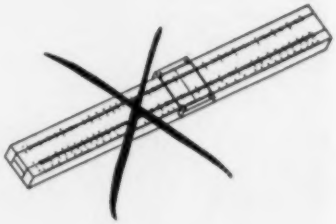

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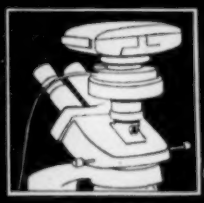

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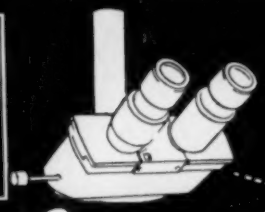

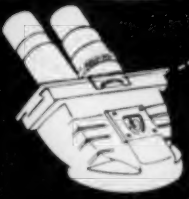

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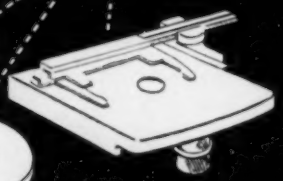

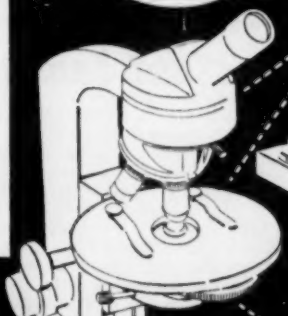

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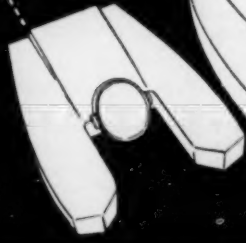

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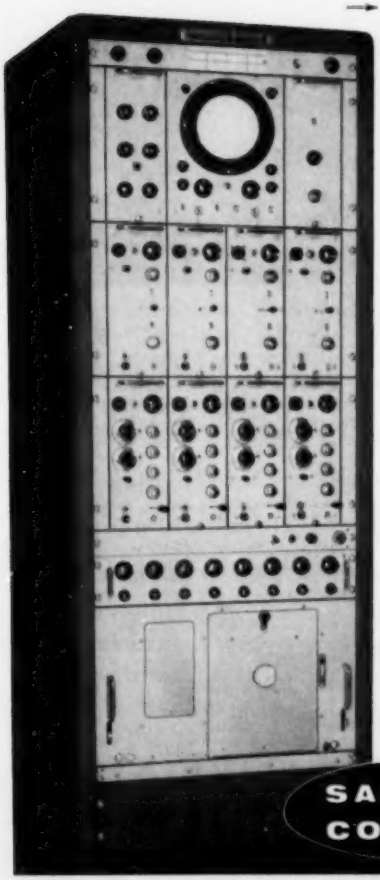

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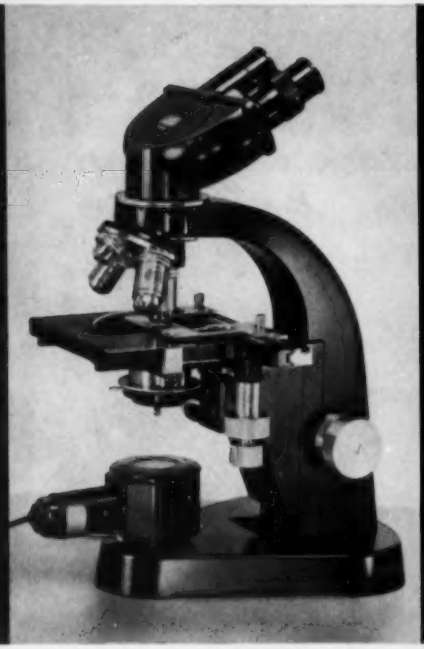

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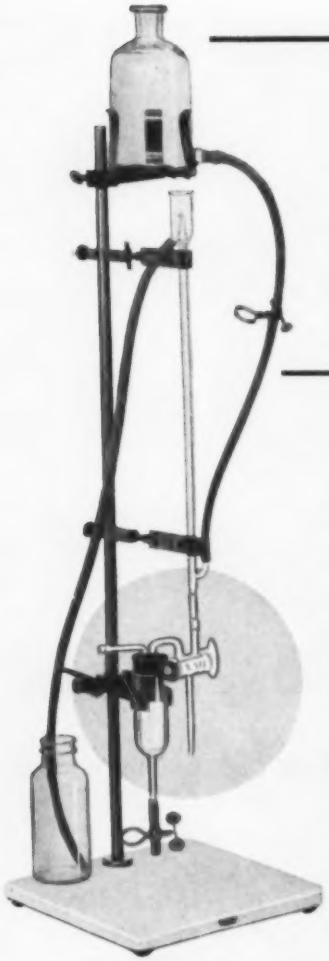

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In use, the pipette is attached to the dispensing burette by means of the upright arm of the stopcock, and to a suction device by means of the bent tube at the left. A waste trap is inserted into the line prior to the suction connection.

Samples are taken by setting stopcock to connect tip with suction line. A small excess is drawn over into the bent tube to serve as an initial rinse for the pipette wall. The sample is delivered by reversal of the stopcock plug to allow solution in the burette to expel the sample through the tip, and to rinse it clean. Following delivery of the required amount of reagent from the burette, the tip, filled with diluent which keeps it clean, is ready for immediate intake of the next sample.

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VOL. 52 SEPTEMBER, 1958 No. 3

CLINICAL AND EXPERIMENTAL

STUDIES IN IRON TRANSPORTATION AND METABOLISM

XI. CRITICAL ANALYSIS OF MUCOSAL BLOCK BY LARGE DOSES OF INORGANIC IRON IN HUMAN SUBJECTS

ELMER B. BROWN, JR., M.D.,* REUBENIA DUBACH, PH.D., AND CARL V. MOORE, M.D.
ST. LOUIS, MO.

SINCE iron is avidly conserved by the mammalian organism and is lost from the body only in small amounts except by hemorrhage, the quantity absorbed is of great importance in controlling the total amount of body iron. The mechanisms which regulate absorption of the metal, however, are largely unknown. Available evidence indicates that absorption is greater than normal when iron deficiency exists[1-3] or erythropoiesis is accelerated.[4, 5] Conversely, it is depressed when storage iron is increased,[4] erythropoiesis is depressed,[4] or when the intestinal tract is satiated by antecedent doses of iron given orally.[6, 7] This latter observation, made on a surprisingly small number of dogs, has given rise to the concept that a "mucosal block" mechanism is a prime regulator of iron absorption. The suggestion proved attractive and has come to be widely accepted even though it fails to explain the excessive absorption and heavy tissue iron deposits found in some subjects after prolonged ingestion of large amounts of dietary or medicinal iron.[8-12] Furthermore, even normal amounts of iron in the diet of patients with idiopathic hemochromatosis are absorbed to a greater degree than normal.[13]

The concept of "mucosal block" originated from acute experiments performed by Hahn and his associates[6] on dogs made anemic by repeated bleeding.

From the Department of Internal Medicine, Washington University School of Medicine, St. Louis, Mo.

These studies were supported in part by Research Grant H-22 from the National Heart Institute and by endowment funds established in memory of Mark A. Edison and L. Russell Kelce.

Received for publication Feb. 21, 1958.

*National Research Council Fellow in Medical Sciences.

The procedure was to feed 100 mg. of ferric iron, give an oral test dose of radioactive iron several hours later, and measure its absorption in terms of the amount of isotope utilized in the synthesis of new hemoglobin. Stewart and co-workers[7] extended Hahn's observations, using a refined technique—again on iron-depleted dogs. Both groups found that the absorption of a standard test dose of radioiron was decreased ("blocked") when preceded by the feeding of a large dose of inert iron several hours earlier. Hahn postulated that an acceptor substance in the intestinal mucosal cell became saturated with iron and suggested that the acceptor might be ferritin. This hypothesis has been elaborated by Granick.[14, 15]

Recently, however, Wöhler and associates[16] have obtained evidence that a high ferritin content of the duodenal mucosa does not cause a "block" to absorption. They gave a single 15 mg. dose of ferrous iron to guinea pigs and noted a progressive increase for 20 hours in the ferritin content of the duodenum. The values then declined and returned to normal in approximately 40 hours. Simultaneous determination of ferritin and nonferritin iron in the liver showed a constant increase up to 20 to 40 hours, which the authors interpret as indicating continuing absorption. In long-term experiments, 15 mg. of ferrous iron were given daily for 28 days. An increase in liver iron was demonstrated even when ferritin values in the stomach, duodenum, and jejunum were maximal, again indicating continuing absorption. After 14 days, there was no further increase in liver iron even though there was a decrease during this period of the ferritin in the mucosal cells of stomach, duodenum, and small intestine. They concluded that a ferritin-mediated block of iron absorption does not exist, even though they accepted ferritin as a mediator of absorption per se. If much iron was absorbed, there was much ferritin; if less was taken up, there was less ferritin. Similar results also were obtained in the guinea pig, by Heilmeyer and co-workers.[17]

Whatever the mechanism, there are certain other observations which may be interpreted as indicating the presence of a "mucosal block." Finch and his co-workers[18, 19] showed that administration of ferrous sulfate by mouth to 2 normal men for 6 and 12 months did not appreciably increase body iron stores as measured by subsequent phlebotomies. These subjects rapidly became anemic when subjected to phlebotomies, whereas other normal subjects given ferrous sulfate orally during the period of blood loss were better protected against anemia. It appeared that iron was not absorbed and stored in substantial amounts during the period before phlebotomies, but that its absorption was increased during the period of anemia. The augmented absorption of iron which occurred during the period of anemia did not continue, however, after the hemoglobin level had been restored to normal, even though iron stores were still depleted as shown by a second course of phlebotomies. Some control of iron absorption seems, therefore, to restrict iron accumulation in the nonanemic subject.

Similarly, iron absorption by animals is depressed when tissue stores are increased by the parenteral administration of iron.[4, 20] Under these circumstances, the ferritin content of the duodenum has been shown to be elevated.

At first glance, this fact appears to suggest that the ferritin may be "blocking" absorption, but the increased ferritin is actually deposited in the submucosa rather than in mucosal cells.[16]

Because the "mucosal block" theory fails to explain the relatively good absorption seen during accelerated erythropoiesis and in occasional subjects who ingest large amounts of iron, and because the role of ferritin as a mediator of the block has been so effectively questioned, many students of iron metabolism have come to believe that another regulating mechanism of iron absorption exists even though its nature remains totally unknown. Care must be taken against assuming that the effect of large doses of orally administered iron in depressing absorption of a tracer dose given several hours thereafter is the same as the depressing effect on absorption of heavy tissue stores, decreased erythropoiesis, or the prolonged oral administration of moderate doses. The circumstances cannot necessarily be equated and confusion has resulted from application of the term "mucosal block" to these several different situations.

For these reasons, a more thorough study of the "blocking" effect of large oral doses of iron was undertaken. Observations were made on human subjects because the importance of this type of "block" had not been tested in man. Findings were not uniform: they varied considerably from subject to subject and at different times in the same individual. In general, the results showed: (1) 2 to 4 mg. of iron per kilogram of body weight, given several hours before a 5 mg. tracer dose of radioiron, did tend to decrease absorption of the tracer dose. (2) The "block" persisted for about 6 hours in normal subjects but only for about 3 hours in iron deficient patients. (3) Ferric chloride tended to decrease absorption of the tracer dose less than did ferrous sulfate. (4) Oral administration of ascorbic acid did not consistently reduce the "block." (5) The nature of the "blocking" effect was not discovered. Saturation of the plasma transferrin did not seem to be important in its production.

PROCEDURE

In each experiment the subject took by mouth an "unblocked" or "control" test dose of radioactive iron and (in a second study) a comparable dose of radioiron 3 or more hours after a large *blocking dose* of inert iron. Three or four experiments were done on almost every subject in an effort to define the conditions which would produce a block. The order in which the different doses were given was varied from subject to subject.

The *blocking interval* was the time between the blocking dose and the test dose. An interval of 6 hours was first selected because Stewart and associates[7] had observed a maximum block at approximately this time. Blocking intervals of 1½, 3, and 4 hours were also employed. The *test dose* was an accurately measured volume of a solution of high specific activity $Fe^{59}SO_4$* giving approximately 1,000,000 counts per minute and containing 5 mg. of Fe (added as crystalline ferrous sulfate). The *blocking dose* was a solution of $FeSO_4$ or $FeCl_2$. Since solutions of ferrous sulfate become oxidized on standing, the calculated amount of crystalline $FeSO_4 \cdot 7H_2O$ was dissolved in water just before it was ingested. These iron salts were chosen as representative of common divalent and trivalent iron compounds. The blocking dose of $FeSO_4$ was either 2 or 4 mg. of iron per kilogram of the subject's body weight; the amount of $FeCl_2$ was uniformly 2 mg. Fe per kilogram of body weight. These

*The Fe^{59} and Fe^{59} used in this investigation were obtained from the Abbott Laboratories under authorization from the Isotopes Division of the Atomic Energy Commission.

TABLE I. ABSORPTION OF A TRACER DOSE OF $Fe^{59}SO_4$ BY 19 IRON DEFICIENT SUBJECTS AND EFFECT OF A BLOCKING DOSE OF $FeSO_4$ GIVEN THREE HOURS BEFORE THE TRACER

PATIENT	SEX	HB. (GM./100 ML.)	HCT. (%)	S.I./T.I.B.C.* (μG/100 ML.)	TRACER DOSE (MG. Fe)	ABSORPTION OF UNBLOCKED TRACER AS MEASURED BY		BLOCKING DOSE OF $FeSO_4$ (MG. Fe/KG.)	ABSORPTION OF TRACER GIVEN 3 HR. AFTER BLOCKING DOSE	
						Fe^{59} IN BLOOD (% OF DOSE)	FECAL RECOVERY (% ABSORBED)		Fe^{59} IN BLOOD (% OF DOSE)	BY FECAL RECOVERY (% ABSORBED)
1. R. J.	M	13.0	41.5	35/511	5	63	72	--	--	--
2. D. T.	F	9.5	30	23/472	5	50	58	4	16	26
3. L. G.	F	5.6	24	10/405	5	76	76	4	36	35
4. D. K.	F	5.6	18.5	35/439	5	48	62	4	15	--
5. D. D.	F	10.7	34	42/498	5	31	--	4	12	--
D. D.	F	10.7	34	42/498	5			2	5	--
6. S. S.	F	8.9	34	65/495	5	63	--	2	16	--
7. M. L.	F	10.2	32	40/426	5	43	--	2	19	--
8. J. P.	F	6.1	26	14/382	5	28	35	2†	26	32
9. M. B.	F	10.7	35	61/384	5	40	42	2†	28	30
10. C. M.	F	8.0	30.5	14/427	5	29	31	--	--	--
11. Z. D.	F	7.8	27.5	9/459	5	24	35	--	--	--
12. A. D.	F	10.0	31	27/457	5	50	62	--	--	--
13. L. J.	F	7.7	26.5	15/365	5	31	33	--	--	--
14. A. B.	F	8.1	25	29/361	5	53	60	--	--	--
15. L. C.	F	5.7	23	18/377	5	45	--	--	--	--
16. E. S.‡	F	11.1	35	22/471	5	30	--	4	38	--
17. B. H.	F	7.6	26	37/467	5	48	--	--	--	--
18. Al. D.	F	9.7	32	----	56.7 mg. Fe (1 mg./Kg.)	56	--	--	--	--
19. I. P.	F	7.9	27	----	82.3 mg. Fe (1 mg./Kg.)	41	--	--	--	--
				Range of absorption (17 subjects)		24 to 76%		Range (10 experiments)	5 to 38%	
				Mean ± standard error (17 subjects)		44.2§ ± 3.5%		Mean ± S.E. (10 experiments)	21.1 ± 3.4%	

Of the 19 patients whose control absorption values are listed, 9 served for a 3-hour ferrous sulfate block (D. D. in 2 experiments). Data from blocking experiments on the others are shown in Tables II and III.

*S.I. = serum iron; T.I.B.C. = total iron-binding capacity of serum.

†Blocking dose given as $FeSO_4$ tagged with Fe^{59}.

‡Huge hiatus hernia.

§For calculation of this average figure, results on Subjects 18 and 19 were not included because of the difference in dose.

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dosage levels were selected as being large enough to ensure an adequate blocking effect, yet small enough to be tolerated by most subjects. The term *blocked absorption* refers to the per cent of a *test dose* of radioiron absorbed after a previous *blocking dose* had been given: *control* or *unblocked absorption* refers to the per cent of a *test dose* of radioiron absorbed when no *blocking dose* had been given.

MATERIAL AND METHODS

The individuals who served as volunteers for the study were (1) healthy medical students or members of the laboratory staff and (2) patients with iron deficiency anemia.

The *blocking dose* of 2 or 4 mg. Fe per kilogram was usually given between 8 and 9 A.M. after an overnight fast. Fasting was continued until the *test dose* of $Fe^{59}SO_4$ (containing 5 mg. Fe) was given, and for 2 hours thereafter. Black coffee was allowed if desired. When the tracer dose was given without a previous blocking dose it was given to the fasting subject early in the morning. When a second dose of radioiron was to be given, at least 2 weeks were allowed to elapse in order to give time for the complete utilization of the first dose. A "base line" blood sample was drawn just before the second dose and assayed for radioactivity with subsequent samples so that the "base line" counts could be deducted.

Absorption of the isotope by normal subjects was measured by determining the difference in per cent between the radioiron ingested and the unabsorbed radioactivity recovered in the feces. Fecal collections were made quantitatively for 7 or more days. *Utilization* of the absorbed iron was measured by determining the per cent of the ingested Fe^{59} which appeared in the circulating blood as newly synthesized hemoglobin. In most of the studies on iron deficient patients, only the Fe^{59} in the blood was determined, since iron deficient subjects utilize absorbed iron almost quantitatively for hemoglobin synthesis.[21]

Radioactive iron in both feces and blood was measured by counting the gamma rays with a well-type scintillation counter.* The feces were collected for 7 days or until the radioactivity of a day's specimen was found to be 1 per cent or less of the administered dose. One hundred or more grams of feces were homogenized with water in a Waring blendor and aliquots of the homogenate (of approximately 3 ml. volume) were weighed in plastic tubes. Triplicate samples were counted and the radioactivity was compared with that of a standard Fe^{59} solution counted at the same time. Blood was drawn by venepuncture into oxalated tubes. Duplicate 3 ml. samples were pipetted into plastic tubes and were hemolyzed with saponin before counting. For the calculation of total radioiron in the circulating hemoglobin, the blood volume was assumed to be 70 ml. per kilogram of body weight except in 6 cases, in which it was measured by the Evans blue dye technique.

In 4 studies the iron of the blocking dose was labeled with Fe^{55} and the tracer dose labeled with Fe^{59}. The Fe^{55} was measured with a Geiger tube with a mica window and a beryllium filter, as recommended by Peacock and associates.[22] Aliquots of the fecal homogenates and of the whole blood were wet ashed with sulfuric acid and the iron was electroplated from the digest onto copper disks for counting.[2] The Fe^{59} in the specimens was measured in the scintillation well counter, which is not sensitive to the soft x-rays of the Fe^{55}.

On each subject, serum iron,[23] iron binding capacity,[24] red blood cell counts, hemoglobin estimations, and packed cell volumes were determined at the beginning of the study and several times subsequently.

Analysis of the results was carried out by the "t" test, calculation of the correlation coefficient, and the analysis of variance.[25]

RESULTS

I. Characteristics of the Block.—

A. *Iron Deficient Subjects.*—

1. *Unblocked absorptions:* The results of unblocked absorption studies on 19 iron deficient subjects are tabulated (Table I). Absorption of the *test dose*

*Manufactured by the Nancy Wood Counter Laboratory, Chicago, Ill.

of radioiron by the 17 iron deficient patients who received 5 mg. of iron as $Fe^{59}SO_4$ averaged 44.2 per cent with a standard error of ±3.5 per cent (range 24 to 76 per cent). There was no significant correlation between the degree of iron absorption and the hemoglobin, hematocrit, serum iron, or unsaturated iron binding capacity values.

2. *Effect of the two different blocking doses (2 mg. and 4 mg. per kilogram) of $FeSO_4$:* Since two different blocking doses of iron were used in these iron deficient subjects, results were analyzed to determine whether the two doses caused different degrees of block. Four observations of blocked and unblocked absorption were made at each dosage level with a 3-hour blocking interval (Table I). Analysis of variance of the data indicated a significant block for both blocking doses of $FeSO_4$ ($p < 0.01$) but no significant difference between the two dosages.

3. *Effect of different blocking intervals on the blocking effect of $FeSO_4$:* When the interval between the blocking dose of ferrous sulfate and the tracer dose was increased to more than 3 hours, the blocking effect tended to diminish or disappear (Table II). Calculation of the correlation coefficient relating the per cent of control absorption to the length of the blocking interval revealed a significant correlation ($r = 0.67$ $p < 0.01$).

TABLE II. EFFECT OF DIFFERENT BLOCKING INTERVALS ON THE ABSORPTION OF RADIOIRON BY IRON DEFICIENT PATIENTS WHEN A BLOCKING DOSE OF $FeSO_4$ WAS GIVEN

	PATIENT	ABSORPTION OF Fe^{59} (% OF DOSE)		BLOCKING DOSE OF $FeSO_4$	PER CENT OF CONTROL ABSORPTION $\left(\frac{\text{BLOCKED ABSORP.}}{\text{UNBLOCKED ABSORP.}} \times 100\right)$
		CONTROL	BLOCKED	MG. Fe/KG.	
A. Blocking interval 1½ hr.	Al. D.*	56	17	5	30
	I. P.*	41	20	4	49
B. Blocking interval 3 hr.	Average of 10 experiments (see Table I)	44	21	2 or 4	48
C. Blocking interval 4 hr.	L. J.	31	21	2	68
	A. B.	53	38	2	72
D. Blocking interval 6 hr.	R. J.	63	71	4	113
	D. T.	50	40	4	80
	E. S.	30	26	4	87

*Al. D. and I. P. each received a tracer dose of 1 mg. Fe per kilogram. All the other subjects received 5 mg. Fe in the tracer dose.

4. *Ineffectiveness of $FeCl_3$ in producing a block in iron deficient subjects:* Ten subjects were given ferric chloride as a blocking agent, and the degree of block was measured at intervals of 3, 4, and 6 hours (Table III). There was no significant correlation between the degree of block and the length of the blocking interval. The data were then pooled and analyzed for degree of block by the paired "t" test. This test revealed that, in iron deficient subjects, no significant blocking effect was produced by ferric chloride, in a dose of 2 mg. Fe per kilogram, given 3 to 6 hours before the tracer dose ($p > 0.50$).

TABLE III. EFFECT OF DIFFERENT BLOCKING INTERVALS ON THE ABSORPTION OF RADIOIRON BY IRON DEFICIENT PATIENTS WHEN A BLOCKING DOSE OF $FeCl_3$ (2 MG./KG.) WAS GIVEN

	PATIENT	ABSORPTION OF Fe^{59} (% OF DOSE)		PER CENT OF CONTROL ABSORPTION $\left(\frac{\text{BLOCKED ABSORP.}}{\text{UNBLOCKED ABSORP.}} \times 100\right)$
		CONTROL	BLOCKED	
A. Blocking interval 3 hr.	J. P.	28 (35)*	38 (44)*	136
	M. B.	40 (42)*	28 (30)*	70
	C. M.	29	22	76
	Z. D.	24	26	108
	A. D.	50	45	90
	L. C.	45	44	98
B. Blocking interval 4 hr.	R. J.	63	42	67
	D. K.	48	34	71
C. Blocking interval 6 hr.	C. M.	29	27	93
	A. D.	50	50	100

*Figures in parentheses represent Fe^{59} absorption as measured by fecal recovery.

5. *Delineation of degree of block:* We have demonstrated that block to iron absorption in iron deficient subjects occurs to a significant degree in these experiments when ferrous sulfate is given in doses of 2 mg. or more of iron per kilogram of body weight and with a blocking interval of 3 hours. The degree of block under these circumstances was determined in 10 experiments. The mean absorption of a blocked dose of iron was 21.1 per cent with a standard error of ±3.4 per cent; the mean unblocked absorption of a comparable test dose by 17 iron deficient patients was 44.2 per cent with a standard error of ±3.5 per cent (Table I). Expressed in another way, the mean blocked absorption of iron deficient patients was approximately 48 per cent of the mean control absorption. At the 3-hour interval, there was no significant correlation between the degree of block and the magnitude of the control absorption ($r = -0.37$; $p > 0.1$).

6. *Effect of decreasing the blocking dose (1 subject):* An attempt was made to determine the minimum dose of iron which would be effective in blocking the absorption of a tracer dose by an iron deficient woman (D. D.). Her unblocked absorption of a 5 mg. test dose of radioiron as $FeSO_4$ was found to be 31 per cent. Then the blocking effectiveness of successive 4, 2, and 1 mg. per kilogram doses of iron as $FeSO_4$, with a constant 3-hour blocking interval, was determined. The results of this experiment are shown graphically (Fig. 1). The 4 mg. per kilogram blocking dose reduced absorption of the tracer to 12 per cent; the 2 mg. per kilogram block reduced absorption to 5 per cent; the 1 mg. per kilogram block resulted in only a slight reduction below the control value—24 per cent, as compared to 31 per cent. These results suggested that (at least in this one iron deficient subject) a definite threshold for block might exist. The fact that there is not a linear relation between the size of the blocking dose and the degree of block is probably due to variation in the individual's susceptibility to a "block" at different times. It has been shown, in a larger population of iron deficient subjects, that (with $FeSO_4$, at a 3-hour blocking interval) there is no significant difference in the blocking effectiveness of doses of 2 and 4 mg. Fe per kilogram.

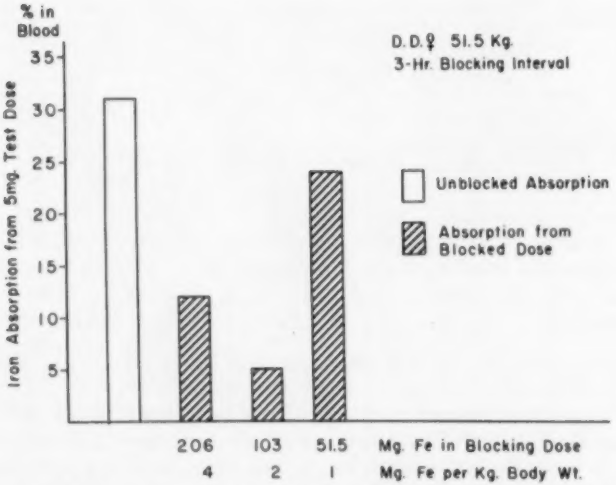

Fig. 1.—The effect of progressively smaller blocking doses of ferrous sulfate on the absorption of a test dose of radioiron by an iron deficient patient. Each column shows the per cent of the tracer dose (5 mg. Fe as $FeSO_4$) absorbed. The white column shows the absorption when no blocking dose had been given; the 3 striated columns show the absorption of the tracer when given 3 hours after blocking doses of (1) 4 mg. Fe per kilogram, (2) 2 mg. Fe per kilogram, and (3) 1 mg. Fe per kilogram.

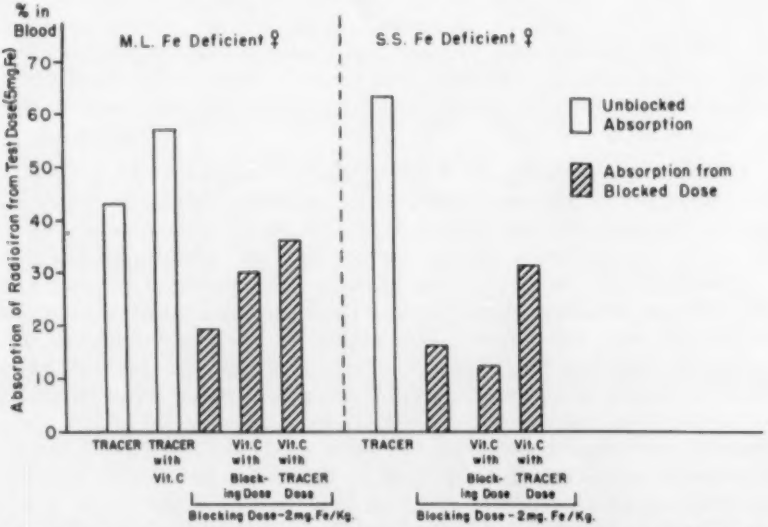

Fig. 2.—The effect of a supplement of one gram of ascorbic acid on the absorption of unblocked and blocked test doses of radioiron by two iron deficient subjects. The blocking dose of $FeSO_4$ (2 mg. Fe per kilogram) was given 3 hours before the tracer. The white columns show the absorption of the tracer when no previous blocking dose had been given. The striated columns show the absorption of the tracer after a blocking dose (given alone or with ascorbic acid). The column farthest to the right, in each study, shows the absorption of a "blocked" tracer dose when ascorbic acid was given with the tracer.

7. *Effect of ascorbic acid on the block (2 subjects):* The effect of ascorbic acid upon the blocking phenomenon was observed in 2 iron deficient subjects. In the first subject (M. L.) unblocked absorption of the tracer dose was 43 per cent when the iron was given alone and 57 per cent when it was given with 1 Gm. of ascorbic acid. A 2 mg. per kilogram blocking dose of iron, given as $FeSO_4$ at a 3-hour blocking interval, produced a definite block (19 per cent absorption). When, however, 1 Gm. of ascorbic acid was given with a blocking dose of the same size, absorption was increased from 19 to 30 per cent. When, in a fourth experiment, this patient was given ascorbic acid with the *tracer* dose rather than with the *blocking* dose, the absorption of the iron was 36 per cent (Fig. 2).

The second subject (S. S.) absorbed 63 per cent of her unblocked tracer dose of iron. A 2 mg. per kilogram blocking dose of iron as $FeSO_4$, given 3 hours before the tracer, reduced absorption from 63 to 16 per cent. One gram of ascorbic acid, when added to the *blocking dose* in another experiment, had no effect in overcoming the block—her absorption was 12 per cent; but another one gram dose of ascorbic acid, when added to the *tracer,* increased absorption of the blocked dose to 31 per cent. The results for both patients are shown graphically (Fig. 2).

The experiments in these 2 iron deficient patients suggest that administration of one gram of ascorbic acid with the tracer dose of iron may in some instance improve absorption of the metal, regardless of whether a prior blocking dose has been given. When given with the blocking dose, ascorbic acid had no consistent effect on the absorption of the tracer dose administered 3 hours later.

8. *Effect of multiple blocking doses (different conditions of block):* Four iron deficient patients were studied to determine the effectiveness of multiple blocking doses of iron, given over a period of 24 hours or longer. The *test dose* of radioiron used in this study was 1 mg. Fe per kilogram of the patient's body weight and was given as ferrous sulfate. To the first patient (L. K.) a blocking dose of 40 mg. Fe as $FeSO_4$ in solution was given every 3 hours for 24 hours; the last dose was given 3 hours before the *tracer dose* of radioactive iron. No block could be demonstrated. In two other studies, 52 and 130 mg. blocking doses of iron as ferrous sulfate were given every 3 hours for 24 hours to iron deficient subjects and were likewise ineffective in producing a block. The 130 mg. dose was administered in tablet form to A. S.; even though it was equivalent to 2 mg. Fe per kilogram, the time necessary for disintegration of the tablets makes it unlikely that the concentration of iron in solution within the upper intestinal tract was ever as great as if the ferrous sulfate had been given in liquid form. Finally, in a fourth study a solution of 51 mg. of iron as $FeSO_4$ was administered to an iron deficient woman (G. S.) daily for 4 days before the tracer dose and reduced her absorption of the tracer to approximately 50 per cent of the control value. The results of these experiments are tabulated (Table IV).

It is unusual that "control" absorption values in 3 of these 4 subjects were less than 15 per cent of the 1 mg. per kilogram test dose of iron as $FeSO_4$.

In another series of iron deficient subjects the average absorption of a dose of this size was 47 per cent.[3] In only one patient (L. K.) was there an explanation for this low absorption, namely, an intercurrent cellulitis, which may have reduced the *utilization* of the absorbed iron and resulted in an erroneously low figure for *absorption* (since absorption was measured by blood radioiron alone). These data suggest (1) that the doses of iron employed to produce a block by a multiple dose schedule were below an effective blocking threshold and (2) that the size of individual blocking doses is more important in producing a block than the cumulative effect of multiple doses.

TABLE IV. EFFECT OF MULTIPLE BLOCKING DOSES ON THE ABSORPTION OF A STANDARD TEST DOSE OF RADIOIRON BY IRON DEFICIENT SUBJECTS*

SUBJECT	HEMOGLOBIN (GM./100 ML.)	HEMATOCRIT (%)	ABSORPTION OF Fe^{59} % OF DOSE		CONDITIONS OF BLOCK
			CONTROL	"BLOCKED"	
1. L. K.	5.1	19	11	12	40 mg. Fe as $FeSO_4$ solution every 3 hours for 24 hr.
2. C. G.	6.4	26.5	13	11	52 mg. Fe as $FeSO_4$ solution every 3 hours for 24 hr.
3. A. S.	9.0	33	40	41	130 mg. Fe as $FeSO_4$ tablets every 3 hr. for 24 hr.
4. G. S.	10.4	38.5	14	7	51 mg. Fe as $FeSO_4$ solution daily for 4 days

*The test dose was 1 mg. Fe per kilogram given as $Fe^{59}SO_4$.

TABLE V. ABSORPTION OF RADIOACTIVE IRON BY NORMAL SUBJECTS; TRACER DOSE OF 5 MG. OF Fe AS $Fe^{59}SO_4$

SUBJECT	SEX	HB. (GM./100 ML.)	HCT. (%)	S.I./T.I.B.C.* (μG/100 ML.)	ABSORPTION AS MEASURED BY	
					Fe^{59} IN BLOOD (%)	FECAL RECOVERY (%)
1. E. B.	M	15.2	43.0	127/280	5.4	7.8
2. B. J.	M	17.9	50.0	126/378	1.7	7.1
3. F. K.	M	16.4	47.5	107/325	15.2	22.8
4. J. S.	M	18.2	49.5	118/253	8.6	15.9
5. D. A.	M	17.8	52.5	102/277	9.3	16.9
6. K. S.	M	13.6	40.0	123/243	5.7	9.0
7. G. T.	M	18.1	49.0	138/291	3.9	7.9
8. E. M.	M	16.1	48.0	126/317	13.0	24.0
9. M. M.	F	13.6	39.5	73/279	27.2	25.7
10. R. G.	M	15.8	45.5	92/263	17.3	25.5
11. B. H.	F	13.5	40.0	80/242	9.4	8.3
12. D. W.	M	16.3	47.0	103/193	9.6	13.7
13. T. A.	M	15.1	44.5	73/279	5.9	19.0
14. W. M.	M	15.8	46.0	122/221	19.0	32.0
				Range	1.7 to 27.2	7.1 to 32
				Mean ± S.E.	10.8 ± 1.8	16.8 ± 2.2

*S.I. = serum iron; T.I.B.C. = total iron-binding capacity of serum.

B. *Normal Subjects.*—

1. *Unblocked absorption:* Unblocked iron absorption was studied in 14 normal subjects (Table V). When a *test dose* of radioiron (5 mg. Fe as $FeSO_4$) was given, *absorption* averaged 16.8 per cent, with a standard error of ± 2.2

per cent (range 7.1 to 32 per cent) when measured by the fecal recovery method; and *utilization* averaged 10.8 per cent with a standard error of ±1.8 per cent (range 1.7 to 27.2 per cent) as measured from the radioiron in the hemoglobin. There was no significant correlation between iron absorption and hemoglobin, hematocrit, serum iron, or unsaturated iron binding capacity in these normal subjects.

2. *Blocking effect of ferrous sulfate:* Table VI records the data on the blocking effect of 2 and 4 mg. per kilogram doses of ferrous sulfate given from 3 to 6 hours prior to the tracer dose. In order to compare data on different subjects, the per cent of the control (unblocked) absorption was calculated as:

$$\frac{\text{"Blocked" absorption}}{\text{"Unblocked" absorption}} \times 100$$

Inspection of the data demonstrates: (1) that a block was not produced in all instances, (2) that there was no consistent difference between the effect of the two blocking doses, and (3) that there was no consistent difference in results when the interval between the blocking and the tracer doses was varied from 3 to 6 hours.

Statistical analysis of the data revealed no significant correlation between the blocking interval and the degree of block, regardless of whether absorption was measured by blood utilization or fecal recovery.

3. *Effect of ferrous sulfate and ferric chloride as blocking agents in normal subjects:* In 3 normal subjects, the blocking effect of ferrous sulfate and ferric chloride was compared at a dose level of 2 mg. Fe per kilogram of body weight, given 3 hours prior to the tracer dose (Table VII). In M. M. a greater blocking effect was observed with ferric chloride than with ferrous sulfate; in the other two subjects the difference in the effects of the two blocking agents was small.

4. *Effect of ascorbic acid:* Ascorbic acid in doses of 500 mg., dissolved in 200 ml. of orange juice, was given to 6 normal subjects at hourly intervals between the blocking and tracer doses of ferrous sulfate (Table VIII, A). Although in two instances the resulting block was of lesser degree, evaluation of the results by analysis of variance showed no significant difference.

It was thought that the fluid ingested with the multiple doses of ascorbic acid might have altered iron absorption by causing the iron to be washed through the small intestine, and that a single large dose might avoid this objection. Accordingly, additional observations were made on 4 subjects. Two men who had received the preliminary blocking dose of iron were given, after a 6-hour interval, one gram of ascorbic acid with the tracer dose. One (F. K.) showed a diminution of the block; the other (J. S.) showed no significant change in the block. A third subject (B. J.) received one gram of ascorbic acid 3 hours after the blocking dose of iron (midway between the blocking dose and the tracer dose) and showed improved absorption of the tracer iron. A fourth subject (M. M.) was given 500 mg. of ascorbic acid with the blocking dose. Blocked absorption was less when ascorbic acid was given than when it was omitted.

TABLE VI. MEASUREMENT OF THE BLOCK TO ABSORPTION OF RADIOACTIVE IRON IN NORMAL SUBJECTS

SUBJECT	UNBLOCKED ABSORPTION* AS MEASURED BY		BLOCKING DOSE OF $FeSO_4$ MG. Fe/KG.	BLOCKING INTERVAL (HR.)	BLOCKED ABSORPTION* AS MEASURED BY		PER CENT OF CONTROL ABSORPTION	
	Fe^{59} IN BLOOD (%)	FECAL RECOVERY (%)			Fe^{59} IN BLOOD (%)	FECAL RECOVERY (%)	BLOOD	FECAL RECOVERY
M. M.	27.2	25.7	2	3	9.4	15.0	35	58
R. G.	17.3	25.5	2	3	17.5	20.5	101	80
B. H.	9.4	8.3	2	3	2.0	3.1	21	37
D. W.	9.6	13.7	2	3	2.4	9.9	25	72
T. A.	5.9	19.0	2	3	14.7	25.8	250	136
E. M.	13.0	24.0	4	4	9.6	15.9	74	66
E. B.	5.4	7.8	4	6	6.4	14.3	118	183
B. J.	1.7	7.1	4	6	1.7	4.3	100	61
F. K.	15.2	22.8	4	6	3.8	7.2	25	32
J. S.	8.6	15.9	4	6	3.4	4.3	40	27
D. A.	9.3	16.9	4	6	4.1	4.7	44	28
K. S.	5.7	9.0	4	6	5.5	14.2	96	158
G. T.	3.9	7.9	4	6	1.3	4.0	33	51
G. T.	3.9	7.9	4	6	1.2	2.4	31	30
R. G.	17.3	25.5	2	6	5.0	9.0	29	35
T. A.	5.9	19.0	2	6	12.8	29.0	217	153
Range (13 experiments)	1.7 to 27.2	7.1 to 25.7		Range (16 experiments)	1.2 to 17.5	2.4 to 29		
Mean ± S.E.	10.2 ± 1.9	15.7 ± 2.0		Mean ± S.E.	6.3 ± 1.3	11.5 ± 2.1		

*The test dose was 5 mg. Fe as $Fe^{59}SO_4$.

TABLE VII. COMPARISON OF THE BLOCKING EFFECT OF FERROUS SULFATE AND FERRIC CHLORIDE IN NORMAL SUBJECTS

SUBJECT	SEX	ABSORPTION MEASURED BY	ABSORPTION OF TRACER DOSE OF 5 MG. Fe AS $Fe^{59}SO_4$		
			CONTROL (%)	AFTER $FeSO_4$ BLOCK* (%)	AFTER $FeCl_3$ BLOCK* (%)
M. M.	F	Fecal recovery	25.7	15.0	8.3
		Hb utilization	27.2	9.4	2.2
B. H.	F	Fecal recovery	8.3	3.1	7.3
		Hb utilization	9.4	2.0	4.1
D. W.	M	Fecal recovery	13.7	9.9	12.4
		Hb utilization	9.6	2.4	3.5

*The blocking dose was 2 mg. Fe per Kg., given 3 hours before the tracer dose.

TABLE VIII. EFFECT OF ASCORBIC ACID ON THE BLOCK PRODUCED BY FERROUS SULFATE IN NORMAL SUBJECTS

DOSAGE AND PATIENT	HOURS	ABSORPTION IN UNBLOCKED CONTROL		BLOCKED ABSORPTION WITHOUT ASCORBIC ACID		BLOCKED ABSORPTION WITH ASCORBIC ACID	
		Fe^{59} IN BLOOD (%)	BY FECAL RECOVERY (%)	Fe^{59} IN BLOOD (%)	BY FECAL RECOVERY (%)	Fe^{59} IN BLOOD (%)	BY FECAL RECOVERY (%)
A. Ascorbic acid 500 mg./hr. between block and tracer							
E. B.	6	5.4	7.8	6.4	14.3	6.8	16.1
B. J.	6	1.7	7.1	1.7	4.3	4.5	4.9
F. K.	6	15.2	22.8	3.8	7.2	2.5	7.3
J. S.	6	8.6	15.9	3.4	4.3	12.1	20.6
D. A.	6	9.3	16.9	4.1	4.7	1.4	8.1
E. M.	4	13.0	24.0	9.6	15.9	13.3	26.5
B. Ascorbic acid 500 mg. with blocking dose. (2 mg. Fe/Kg.)							
M. M.	3	27.2	25.7	9.4	15.0	3.6	7.4
C. Ascorbic acid 1,000 mg. midway between block and tracer							
B. J.	6	1.7	7.1	1.7	4.3	7.2	15.3
D. Ascorbic acid 1,000 mg. with tracer							
F. K.	6	15.2	22.8	3.8	7.2	9.1	20.0
J. S.	6	8.6	15.9	3.4	4.3	1.5	2.3

The blocking dose was 4 mg. Fe per kilogram except in the case of M. M. who received 2 mg. Fe per kilogram. The test dose was 5 mg. Fe as $Fe^{59}SO_4$.

TABLE IX. COMPARISON OF THE ABSORPTION OF BLOCKED AND UNBLOCKED DOSES OF RADIOIRON BY NORMAL SUBJECTS

NO. OF SUBJECTS	CONDITION OF RADIOIRON ADMINISTRATION	ABSORPTION OF TEST DOSE					
		MEASURED BY FECAL RECOVERY			MEASURED BY Fe^{59} IN BLOOD		
		MEAN (%)	STANDARD ERROR (%)	RANGE (%)	MEAN (%)	STANDARD ERROR (%)	RANGE (%)
14	Unblocked*	16.8	±2.2	7.1 to 32.0	10.8	±1.8	1.7 to 27.2
27	Blocked†	11.8	±1.6	2.4 to 29.0	6.0	±0.9	1.2 to 17.5

*Fourteen experiments shown in Table V.

†Includes 16 experiments from Table VI, 3 experiments from Table VII, and 8 experiments from Table VIII, A, B, and C; the experiments from Table VIII, D have not been included.

5. *Delineation of the degree of block:* Since no significant or consistent effect of blocking interval, blocking agent, or ascorbic acid could be demonstrated, all these groups were pooled to evaluate whether or not a significant block to iron absorption occurred in normal subjects. It was assumed that the 2 and 4 mg. Fe per kilogram blocking doses were equally effective in normal subjects, as they had been shown to be in the iron deficient patients. With this pooling of data, 27 observations were available for analysis. It was found that a significant block to iron absorption could be produced in normal subjects both when measured by fecal recovery and when measured by blood utilization ($p < 0.01$). Values comparing the blocked and unblocked absorption are shown in Tables VI to IX and are graphed in Fig. 3.

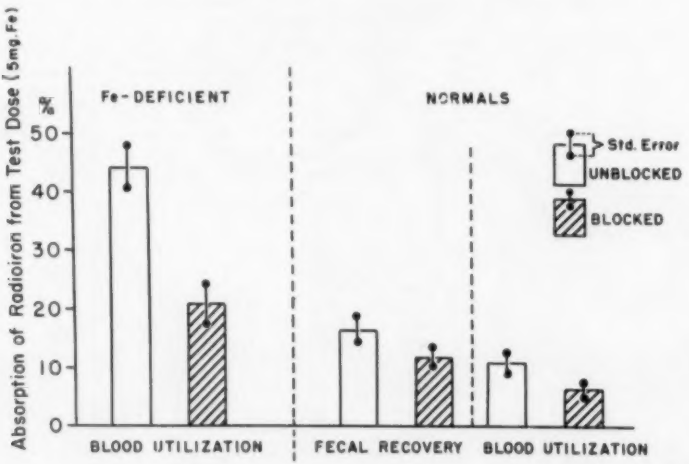

Fig. 3.—A comparison of the mean absorption of a test dose of radioiron when given alone (white columns) and after a "blocking" dose of iron (striated columns) to normal and iron deficient subjects. The blocking dose for each group was either 2 or 4 mg. Fe per kilogram as $FeSO_4$ (except in 3 normal subjects who received 2 mg. Fe per kilogram as $FeCl_3$). The blocking dose was given 3 hours before the tracer to iron deficient subjects and 3, 4, or 6 hours before the tracer to normal subjects.

C. *Comparison of Average Degree of Block in Normal and Iron Deficient Subjects.*—The mean absorption of a "blocked" dose of iron by 27 normal subjects was 11.8 ± 1.6 per cent (as measured by fecal recovery); the mean unblocked absorption of 14 normal subjects was 16.8 ± 2.2 per cent (Table IX). In the series of iron deficient patients the mean absorption (utilization) of a "blocked" dose of iron was 21.1 ± 3.4 per cent; the mean unblocked absorption was 44.2 ± 3.5 per cent (Table I). Expressed in another way, the mean blocked absorption of the normal subjects was 70 per cent of the mean control absorption, and for iron deficient subjects the corresponding figure was 48 per cent.

II. Exploration of the Mechanism of the Block.—The preceding experiments have defined some of the conditions necessary for the production of a block

to iron absorption in the iron deficient and in the normal subject. The experiments to be described were undertaken with the object of exploring the mechanism and site of the block.

A. *Measurement of the Amount of Iron Remaining in the Stomach After Three Hours.*—A possible explanation of the blocking effect of a large antecedent dose of iron is that a portion of the large dose may linger in the stomach until the time when the tracer is given and become mixed with it. Mixing would reduce absorption by diluting the amount of tracer in the gut. Experiments were planned (1) to measure the amount of the blocking dose which was still in the stomach at the end of 3 hours and (2) to determine the effect on absorption of mixing the "blocking" and "tracer" doses before they were ingested.

TABLE X. IRON FOUND IN STOMACH WHEN LAVAGE WAS PERFORMED THREE HOURS AFTER GIVING A "BLOCKING DOSE"

SUBJECT	NORMAL OR IRON DEFICIENT	BLOCKING DOSE (MG.)	Fe OR Fe^{59} RECOVERED BY (GASTRIC) LAVAGE AT 3 HR. (% OF DOSE)
E. B.*	Normal	320 mg. Fe^{++59}	8.38† 7.35‡
E. M.	Normal	280 mg. Fe++	0.04‡
J. B.*	Fe-Def.	109 mg. Fe^{+++59}	0.65† 0.79‡
A. B.	Fe-Def.	111 mg. Fe++	7.2‡

*In the first experiment on E. B. and in the experiment on J. B., the radioiron was mixed with the "blocking dose."
†Fe was measured by radioactivity measurement.
‡Fe was measured chemically.

A normal man, E. B., who had previously taken blocked and unblocked tracer doses of radioiron, took by mouth, in a single dose, 320 mg. of iron as ferrous sulfate (4 mg. Fe per kilogram) tagged with Fe^{59}. At the end of 3 hours a gastric tube was swallowed and the stomach was thoroughly lavaged. The residual iron was determined and found to be 8.4 per cent of the amount ingested.* In other words, of the 320 mg. of Fe taken by mouth, only 8 per cent (26 mg.) was in the stomach and potentially available for mixing with a subsequent dose at the end of 3 hours. At the end of 6 hours presumably even less would have been available for mixing. This subject absorbed 6.8 per cent of the test dose as measured by fecal recovery and 4.1 per cent by blood utilization. A second subject, E. M., was given a 4 mg. per kilogram blocking dose of ferrous sulfate and underwent gastric lavage 3 hours later. Only 0.04 per cent of the iron was recovered in the gastric washings (Table X). Similar experiments were done on two iron deficient patients; the amounts of iron recovered by gastric lavage after a 3-hour interval were 0.7 per cent and 7.2 per cent, respectively (Table X). Of the two subjects who had residual gastric iron values of 7 per cent or over, E. B. had taken black coffee 30 minutes before the

*The iron was determined by measurement of the radioactivity and by chemical analysis. The two methods agreed within 1 per cent.

dose of iron and A. B. had taken a Coca-Cola and some crackers an hour before. It is possible that the beverage and/or food may have slowed gastric emptying in these two subjects.

The foregoing observations suggest that there is no significant gastric mixing of the antecedent blocking dose with the tracer and that mixing alone does not play an important role in producing a block. No attempt was made to determine the residual iron in the duodenum at the site of presumed maximal absorption. Motility characteristics of this portion of the bowel make any large residual there unlikely.

B. *Measurement of Absorption of the Blocking Dose and of the Tracer Dose Simultaneously by the Double Isotope Technique.*—Some insight into the mechanism of the "block" might be gained if the *absorption of the blocking dose* could be measured and compared with the degree of absorption of the tracer. By labeling the blocking dose with one isotope of iron (Fe^{55}) and the tracer dose with another (Fe^{59}) the level of absorption of each dose could be determined. Two normal and two iron deficient subjects were given, in successive experiments, (1) an unblocked test dose of $Fe^{59}SO_4$, (2) a *blocking dose* of $Fe^{55}Cl_3$, followed by a test dose of $Fe^{59}SO_4$, and (3) a *blocking dose* of $Fe^{55}SO_4$, followed by a test dose of $Fe^{59}SO_4$. If a greater block were to be produced consistently by the better absorbed $Fe^{55}SO_4$ than by $Fe^{55}Cl_3$, the result would suggest an intramucosal site for the "block." On the other hand, if there were no correlation between the amount of the blocking dose absorbed and its blocking effect, it would be tempting to believe that simple accumulation of iron salts in the lumen of the gut had served to make the radioiron less available to the absorbing surface.

TABLE XI. COMPARISON BY THE DOUBLE ISOTOPE TECHNIQUE OF ABSORPTION FROM THE TRACER AND THE "BLOCKING" DOSES OF IRON

SUBJECT	ABSORPTION MEASURED BY	ABSORPTION OF TRACER DOSE			ABSORPTION FROM THE BLOCKING DOSE OF	
		CONTROL (%)	AFTER $FeCl_3$ BLOCK (%)	AFTER $FeSO_4$ BLOCK (%)	$Fe^{55}Cl_3$ (%)	$Fe^{55}SO_4$ (%)
D. W. Normal	Fecal recovery	13.7	12.4	9.9	11.6	12.6
	Hb utilization	9.6	3.5	2.4	1.0	5.8
B. H. Normal	Fecal recovery	8.3	7.3	3.1	11.3	14.4
	Hb utilization	9.4	4.1	2.0	2.5	4.2
J. P. Fe-def.	Fecal recovery	34.7	43.8	31.8	9.1	17.1
	Hb utilization	28.1	37.6	25.7	3.2	13.6
M. B. Fe-def.	Fecal recovery	41.8	29.7	29.6	5.5	26.3
	Hb utilization	40.2	28.0	28.0	5.1	13.7

Tracer dose 5 mg. Fe as $Fe^{59}SO_4$.
Blocking dose 2 mg. Fe per kilogram of body weight.
Interval between blocking and tracer doses 3 hours.

Results were not consistent enough to differentiate between these two possibilities. While somewhat better "block" was produced by the blocking dose of ferrous sulfate in 3 instances, there was no clear correlation between the amount of iron absorbed from the blocking dose itself and the decrease in absorption of the tracer dose (Table XI). In the fourth instance (M. B.), there was considerably greater absorption of ferrous than of ferric iron from the blocking

doses, yet the blocking effect on the tracer dose was equal. Interpretation of results is further complicated by the difference in values for absorption of the blocking doses obtained by the fecal recovery and iron utilization methods.

C. *Attempts to "Block" Iron Absorption by the Parenteral Administration of Iron.*—In another study, utilizing a different approach, an attempt was made to block absorption of radioiron in a normal subject by artifically saturating the plasma iron-binding capacity by intravenous injection of saccharated iron oxide. It was reasoned that, if the plasma transferrin were completely saturated with iron, it could not accept iron from the intestinal mucosal cell and that, as a result, iron in the process of absorption might accumulate in the mucosal cell and "block" the further absorption of additional iron. A normal subject, W. M., took by mouth an unblocked tracer dose of iron and his absorption was measured. In a second experiment, 3 hours before the oral tracer dose was to be given, he received saccharated iron oxide (containing 50 mg. Fe) intravenously in order to saturate his iron-binding capacity. Additional injections of 100 mg. Fe were given at the time of the oral dose and 2½ and 5½ hours later, in order to keep the transferrin saturated with iron throughout the absorptive period. The procedure was successful in raising the level of the serum iron to values 5 to 10 times normal and in completely saturating the iron-binding capacity (Table XII). However, no block to iron absorption occurred (as measured by fecal recovery). The chief effect of the parenteral iron was to reduce the *utilization* of the absorbed iron. Only 20 per cent of the absorbed radioiron was utilized for hemoglobin synthesis, as compared to 60 per cent in the control experiment (Table XII). The result of this isolated experiment confirms in a human subject experiments performed on dogs.[26] The combined evidence is at variance with Laurell's hypothesis that the amount of transferrin available to bind iron regulates the amount of iron absorbed.[27] If the parenterally administered iron actually "blocked" the mucosal cell as well as the plasma acceptor for iron, the block was a very inadequate one, since one-third of the oral tracer dose penetrated it.

TABLE XII. ATTEMPT TO BLOCK THE ABSORPTION OF A STANDARD DOSE OF RADIOIRON BY SATURATING THE PLASMA TRANSFERRIN OF A NORMAL SUBJECT, W. M. 5 MG. Fe AS $Fe^{59}SO_4$ WAS GIVEN ORALLY AT THREE HOURS

TIME (HR.)	SERUM IRON μG/100 ML.	U.I.B.C.* μG/100 ML.	SACCHARATED Fe OXIDE I.V. (MG.)
0	123	117	50
3	344	0	100
5½	835	0	100
8½	913	0	100

Results: (a) Control absorption, 32.0 per cent; utilization, 19.0 per cent. (b) "Blocked" absorption, 33.5 per cent; utilization, 6.7 per cent.

*U.I.B.C. = unsaturated iron-binding capacity of serum.

DISCUSSION

The above studies establish the fact that a block to iron absorption can be produced in human subjects by the administration of moderately large doses of iron salts several hours prior to the tracer dose. The block was never complete; it varied considerably in different subjects and was not consistently observed. The reason for the rather striking variations is not known, but

several possible explanations may be offered: (1) the degree of block may be modified at different times by factors which are still obscure; or (2) differences in intestinal motility, gastrointestinal mucus, or pH may alter the time the iron is in contact with the mucosal surface or the chemical changes it undergoes in the intestinal tract. As a result, the effectiveness of the block would be changed. Every subject was employed as his own control, but absorption of an unblocked tracer dose probably also varies from day to day because intestinal motility, etc., cannot be standardized. Even so, statistical analysis demonstrated clearly that a block was produced in most instances. Its duration in normal subjects was approximately 6 hours.

Absorption of the blocked tracer dose averaged 70 per cent of control values for normal subjects and 48 per cent for iron deficient patients (Fig. 3). It is felt, however, that no conclusions about the relative efficiency or degree of block should necessarily be drawn because values for the normal are much lower than for the iron deficient subjects and further depression may be more difficult to detect. The fact that the block is of shorter duration in iron deficient patients (approximately 3 as opposed to 6 hours) may reflect their increased efficiency of uptake and indicate that they "clear" their mucosa more rapidly. That interpretation would favor an intramucosal rather than an intraluminal site for the "block."

Ferric chloride was relatively less effective as a blocking agent than was ferrous sulfate. Since evidence indicates that iron must be reduced to the ferrous form before it can be assimilated, the reduction rate of the ferric chloride in human subjects may be so slow that the necessary concentration of ferrous ions to produce a block may not be achieved. There were several exceptions (Tables III and VII) as one might expect if there were variation in the reduction capacity of the intestinal tract at different times. It will be remembered that dogs are capable of absorbing ferric iron well, presumably because they reduce it more rapidly; in these animals, ferric iron is an effective blocking agent.[6, 7]

Ascorbic acid was added to blocking doses of ferrous sulfate, to the tracer dose, or given between the two to find out whether it would decrease the degree of any block (Fig. 2, Table VIII). Ascorbic acid has been shown to increase the absorption from ferric chloride and from food iron,[28-30] presumably by accelerating the conversion of ferric to ferrous iron. The possibility has never been excluded, however, that it may exert its augmenting effect on iron absorption in some other way, e.g., by forming a chelate which might be taken up by the intestinal mucosa more easily, by increasing transport across cellular membranes, or by aiding the transport of mucosal iron to blood and other tissues. The effect of ascorbic acid was so irregular, however, that no definite conclusions can be drawn. Irrespective of which schedule of ascorbic acid administration was employed, absorption was augmented in only half the cases (Fig. 2, Table VIII); in several instances, assimilation was even decreased.

The conditions responsible for a block of absorption in these studies are somewhat artificial and not likely to be encountered in man except when iron salts are taken medicinally or under unusual circumstances when large amounts of iron are found in beverages or in food (as among Bantus from

cooking in iron pots). Under ordinary conditions of diet, the amounts of iron in food are so small (12 to 20 mg. per day) that they would not be able to produce the kind of block observed in these experiments. Furthermore, absorption from therapeutic doses of iron, or from beverages and food containing relatively large amounts of iron, has been great enough to cause "iron overload."[8-12] Any block which did occur under these circumstances was not great enough to prevent accumulation of abnormally large amounts of the metal. It is of interest that multiple doses of approximately 1 mg. of iron as ferrous sulfate per kilogram of body weight given every 3 hours for one day or at daily intervals for 4 days failed to produce any appreciable block (Table IV). Under these circumstances, the effect was apparently not cumulative. Size of the blocking dose itself seemed to be of more importance than the duration of administration. In addition, as has already been emphasized in the introduction, there has been a tendency in the literature to equate the block produced by large doses of inorganic iron salts with the fact that normal persons assimilate iron less well than do those who are iron deficient. The situations are quite different, and may be—indeed, probably are—the result of entirely different mechanisms.

The degree of block to iron absorption demonstrated in normal subjects is certainly inadequate to prevent the absorption of unneeded iron from the diet. One would have little difficulty, for example, in explaining the large tissue iron accumulation in Bantu natives, whose diet has been estimated to contain as much as 200 mg. of iron per day.[9] Using figures derived from our observations on normal subjects, a "block" in the Bantu would reduce absorption from an expected 34 mg. to 23 mg. This would still yield an increment of iron for shunting to storage depots of 22 mg. in excess of the adult male's estimated daily requirement. From our observations it also seems probable that large amounts of iron may be absorbed and stored by the unfortunate patient who is anemic but not iron deficient and who may be given iron medicinally for a prolonged period in a vain attempt to cure his anemia. These considerations re-emphasize the dangers of indiscriminate iron therapy due to overestimating the safeguard provided by a supposed "mucosal block."

The nature or mechanism of the block was not discovered. The possibility that the results were an artefact produced by retention of a large portion of the blocking dose in the stomach and subsequent mixing with the tracer ferrous sulfate was ruled out. Saturation of the transferrin with parenterally administered saccharated iron oxide did not reduce absorption of iron from the tracer dose. Evidently, neither saturation of the iron-binding capacity nor elevation of the serum iron after the blocking dose was the mechanism whereby absorption of the tracer iron was "blocked." In 4 experiments, the double isotope technique was employed to determine whether increased absorption of the blocking dose (tagged with Fe^{55}) could be correlated with greater degree of block of the Fe^{59} tracer dose (Table XI). No such correlation was evident, even though a surprisingly large amount of the iron in the blocking doses was absorbed. The data, therefore, are not definitive enough to indicate clearly whether the block was intraluminal or within the mucosal cells.

SUMMARY AND CONCLUSIONS

The block to iron absorption produced by large doses of inorganic iron given several hours before a tracer dose tagged with Fe^{59} was studied in iron deficient and in normal human subjects. The results were not uniform: they varied from subject to subject, and also in the same individual, but demonstrated the following general characteristics of the block.

1. Blocking doses of 2 to 4 mg. of iron per kilogram of body weight given several hours before a 5 mg. tracer dose as ferrous sulfate did decrease absorption in most instances although they never blocked assimilation completely.

2. The block was of shorter duration in iron deficient patients (about 3 hours) than in normal individuals, in whom it persisted for about 6 hours.

3. Ferric chloride was less effective than ferrous sulfate in blocking iron absorption.

4. Administration of ascorbic acid did not consistently reduce the block.

5. Saturation of the plasma transferrin or elevation of the serum iron by parenterally administered iron did not cause decreased absorption from the tracer dose.

6. In double isotope experiments with the blocking dose tagged by Fe^{55} and the tracer dose by Fe^{59}, there was no consistent correlation between the absorption from the blocking dose and the degree of block.

The discussion emphasizes: (1) the artificial nature of the block produced by large quantities of inorganic iron; (2) the fact that the amounts of food iron in an ordinary diet are so small that they could not be expected to produce a block of this nature; (3) that this type of block cannot be used as an explanation for the lesser absorption of iron by normal than by iron deficient subjects; (4) that the site and nature of the block were not discovered.

Block to iron absorption occurs when a large dose of an iron salt has been administered several hours before a second "test" dose. This phenomenon is unphysiologic and cannot be equated with the still undiscovered mechanism controlling iron absorption under natural conditions. Experimental evidence does not justify the current use of the term "mucosal block" to describe the physiologic regulation of iron absorption. Until the factors which control iron absorption are more clearly understood, the use of the phrase "mucosal block" should be abandoned.

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ERYTHROCYTE RADIOIRON UPTAKE IN THE RAT AND THE EFFECTS OF COBALT

DONALD R. KORST, M.D., AND FRANK H. BETHELL, M.D.
ANN ARBOR, MICH.
WITH THE TECHNICAL ASSISTANCE OF MRS. ELIZABETH ONG

THE rate of erythropoiesis as measured by incorporation of radioactive iron (Fe^{59}) in hemoglobin is the methodology of this study. Techniques used are patterned after those of Plzak and associates.[1] The turnover of radioiron in the rat affords a rapid and efficient measure of erythropoiesis and a means of studying erythropoietic stimulants or depressants. Tracer methods employing preparations of high specific radioactivity which do not significantly affect the metabolic iron pool have been used to study iron utilization in hemoglobin production by growing rats, iron deficient rats, and following blood loss.[2] More recently the method has been applied in the study of cobalt polycythemia,[3] the effects on erythropoiesis of radiation[4-6] and of thermal injury,[7] as well as the iron turnover in hypophysectomized rats as a means of measuring erythropoietic factors.[8, 9]

MATERIALS AND METHODS

1. Female white (Sprague-Dawley) rats, weighing 150 to 200 grams, are maintained on a Rockland stock diet. They are weighed at the beginning and end of the study. Female rats are preferred as their slower growth facilitates use of uniform weight groups which appears to be important.

2. The test material, adjusted to a volume of 2 ml. per dose, is injected intraperitoneally, subcutaneously, or intravenously each day or twice daily for 2 or 3 days prior to the injection of the radioiron. Penicillin may be added to prevent infection, and Ringer's solution or physiologic saline is used as control test material. The route of administration does not appear to be critical, and the subcutaneous route is the easiest to use.

3. Ferrous (Fe^{59}) citrate in aqueous solution is diluted so that 0.5 ml. contains 2 μc of radioiron. The specific activity is 2,040 μc per gram so that about one microgram of iron will be injected. This is given intravenously and enters the rat plasma iron pool which is about 4 to 5 μg. Using the same tuberculin syringe which is to be employed for all rats, standards are made up by adding 0.5 ml. or 2 μc to a 250 ml. volumetric flask. Three 2 ml. aliquots of each of these standards are measured for radioactivity, and the average is taken as the standard for total injected radioiron. The iron is injected within 4 hours after the last injection of the test material under study. A rubber band tourniquet is employed, the tail is warmed in water, and a No. 25 needle is inserted into a dorsal tail vein.

From the Radioisotope Service, Ann Arbor Veterans Administration Hospital, and the Department of Medicine, University of Michigan School of Medicine, Ann Arbor, Mich.

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4. Blood is taken for hemoglobin measurements and scintillation well counting at 18, 24, and 42 hours after the radioiron injection. The rats are lightly etherized to facilitate working without excessive blood loss. The tail is warmed, and a dorsal vein is nicked followed by withdrawal of 0.02 ml. of blood into a capillary pipette. This is immediately placed in 5 ml. of Drabkin's cyanmethemoglobin solution. The tail is firmly held to prevent further blood loss.

5. After determination of hemoglobin concentration, two 2 ml. aliquots of the same solution are placed into well counter tubes and counted. The activity, as measured in a standard well counter, usually ranges from 100 to 800 c.p.m.

6. The assumed blood volume[10] is taken as the weight in grams (average at beginning and end of study) × 4.59 per cent, expressed in milliliters. In these studies we found hemoglobin values of 14.7 ± 0.9 Gm./100 ml. For purposes of standardization, we have corrected each rat's hemoglobin (average of first, second, and third day values) to 15.0 Gm./100 ml.

Therefore:

(a) $\text{Blood Vol.} = (\text{Ave. Wt.} \times \frac{4.59}{100}) \times \frac{(\text{Ave. Hb.})}{15.0}$

$\text{Blood Vol. (ml.)} = \text{Ave. Wt. (Gm.)} \times \text{Ave. Hb. (Gm.)} \times 0.00306$

(b) $\text{Total injected c.p.m. } Fe^{59} = \text{total injected c.p.m.}$

$\text{Total injected c.p.m.} = \text{standard (2 ml.) c.p.m.} \times 125$

(standard = 0.5 ml. diluted to 250)

(c) $\text{Rat blood activity per ml.} = \frac{1.0 \text{ ml.}}{0.02 \text{ ml.}} \times \frac{5}{2} = 125 \times \text{c.p.m. in 2 ml. sample}$

(d) A final formula to represent the per cent uptake of injected radioiron in the rats' erythrocytes is:

$$\text{Per cent R.B.C. uptake} = \frac{(c) \times (a)}{(b)} \times 100$$

$$= \frac{\text{c.p.m. (2 ml. sample)} \times 125 \times \text{Wt.} \times \text{Ave. Hb.} \times 0.00306}{\text{c.p.m. (2 ml. Std.)} \times 125} \times 100$$

$$= \frac{\text{c.p.m. (2 ml. sample} \times \text{Wt.} \times \text{Ave. Hb.} \times 0.306}{\text{c.p.m. (2 ml. Std.)}}$$

Animals were used in groups of 4 to 6 rats, and radioiron uptake observations were made during 3- to 5-day periods. The amounts of cobalt are expressed as elemental cobalt and were given in the form of the cobaltous chloride hexhydrate ($CoCl_2 \cdot 6\ H_2O$) in solution. Splenectomy was performed on some of the animals under ether anesthesia. The splenic vessels were ligated with as little blood loss as possible. Hypophysectomized rats* were used 10 days or longer after operation. Cobalt polycythemia was induced in rats by giving cobalt, 2 mg. intraperitoneally each day for 25 days, in order to raise the hemoglobin to an average level of 18.5 Gm.

RESULTS

The mean uptake of the tracer at 1, 2, and 3 days is shown in Fig. 1 for full-grown rats and for rats with an average weight of about 180 Gm.[11] The high uptake in the full-grown rats may be due to dietary iron deficiency, but the uptake curve was not affected by feeding iron nor was there demonstration of iron deficiency by measuring plasma serum iron levels of the full-grown rats. Serum iron determinations on both groups revealed a mean serum iron of 122 μg per 100 ml. of the pooled sera of 4 rats, which represented 26 per cent saturation of the total binding capacity of the rat serum. It may be that the full-grown rat has a normal iron turnover which plateaus at 90 to 100 per

*Obtained from the Chicago Hormone Assay Laboratories.

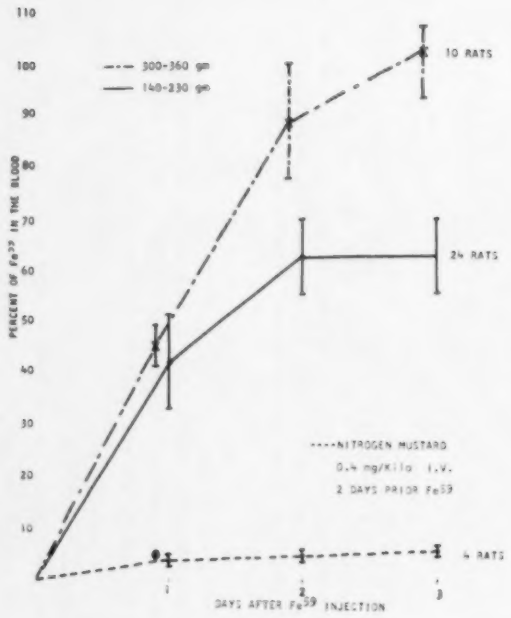

Fig. 1.

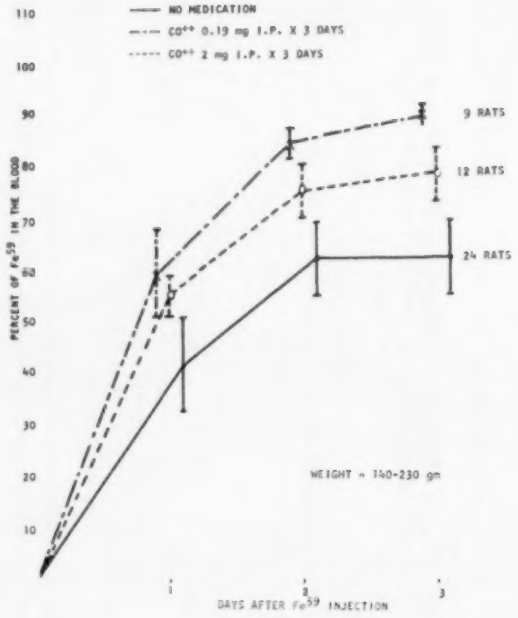
NO MEDICATION
Co++ 0.19 mg I.P. X 3 DAYS
Co++ 2 mg I.P. X 3 DAYS
9 RATS
12 RATS
24 RATS
WEIGHT = 140-230 gm
PERCENT OF Fe59 IN THE BLOOD
DAYS AFTER Fe59 INJECTION

Fig. 2.

Fig. 1.—The effect of weight and nitrogen mustard on the Fe^{59} uptake in male rats. Incorporation of Fe^{59} into hemoglobin during 3 days after intravenous injection of ferrous (Fe^{59}) citrate into rats. The difference between full-grown and medium-sized rats should be noted. The depression of iron incorporation by nitrogen mustard conditioning is also shown. The means and plus or minus one standard deviation are shown.

Fig. 2.—The effect of cobalt on Fe^{59} incorporation into hemoglobin in the rat. A greater increase in Fe^{59} incorporation was produced by the 0.19 mg. dose than by the 2.0 mg. dose of cobalt. Means and plus or minus one standard deviation are shown.

cent in 2 or 3 days. The growing rats in the 180 Gm. range have a more rapid turnover with the plateau at 50 to 60 per cent. With medium-weight rats, mean normal uptakes of about 36 per cent in one day and 50 per cent in two days were observed. The second day value evidently represents a plateau or maximum concentration of the tracer in the circulating hemoglobin, as determinations on the fourth and fifth days after Fe^{59} injection revealed plateaus of between 48 to 55 per cent. The uptake curves are expressed as means of the members of each group of rats, with one standard deviation denoted by the vertical lines.

Administration intravenously of freshly prepared nitrogen mustard (Mustargen B‡) in a dose of 0.4 mg. per kilogram, 2 days prior to the iron injection, severely depressed the uptake of iron or iron turnover as shown in Fig. 1. When 1 mg. per kilogram of nitrogen mustard was given to 4 rats, all the animals died between the third and fourth day. The one rat which survived for 3 days had an iron uptake of 5 per cent shown in Fig. 1 by the symbol.

Fig. 2 presents a summary of 3 experiments designed to determine the acute effects of cobalt on iron turnover. One group was given Ringer's solution intraperitoneally in place of cobalt. Each experiment utilized 4 or 5 rats in a group, and the experiments were conducted simultaneously employing the several doses of cobalt and the control material. The graph represents a summary of these experiments. The 0.19 mg. dose approximates 1 mg. per kilogram of body weight of elemental cobalt, and the 2 mg. dose approximates 10 mg. per kilogram of body weight. The latter amount of cobalt approaches a lethal dose which is in the neighborhood of 12 to 15 mg. of elemental cobalt per kilogram of body weight. It undoubtedly exerts some toxic effects as well as stimulating erythropoiesis. The smaller dose of cobalt apparently exerted a greater effect on iron utilization. The iron turnover appeared to be similarly affected as compared to the normal control group, whether the cobalt had been administered for 25 days or for 3 days as shown in Fig. 3.

Several groups of hypophysectomized rats were studied as shown in Fig. 4. The 24 hour iron uptake of such rats, not receiving cobalt, was about 12 per cent, which is in agreement with values of 10 per cent or lower at 16 and 18 hours as previously reported.[8, 9] There was a gradual rise in uptake to about 30 per cent over the 3-day period. The effect of cobalt on these animals was more striking than on the normal rats as shown by approximately a fourfold increase in the Fe^{59} uptake at 48 hours.

Splenectomy in rats has been said to influence the polycythemia-producing property of cobalt.[12] Several groups of rats were subjected to splenectomy, and the results are shown in Fig. 5. The iron tracer given one day postsplenectomy without any other medication revealed an iron uptake which was greater than that of normal rats, an effect probably attributable to blood loss during surgery. However, another group of rats splenectomized at the same time and given cobalt for 3 days before the iron tracer was injected, demonstrated a significantly higher uptake than either the unoperated control or the splenectomy group indicating that cobalt exerted an effect on the iron turnover in addition to that caused by operative hemorrhage, and that splenectomy probably does not influence the mechanism of the action of cobalt.

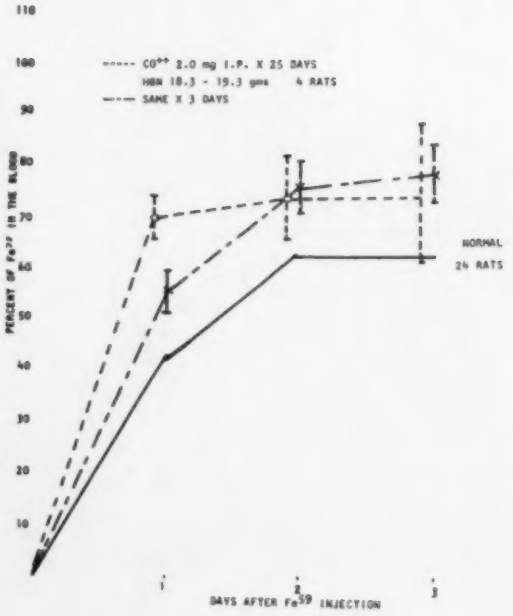

Fig. 3.

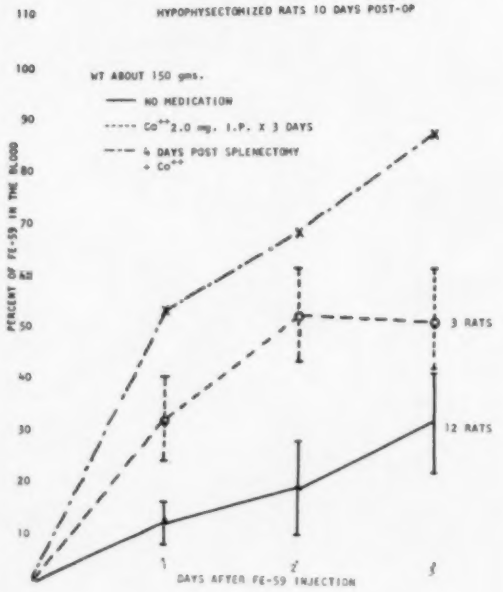

Fig. 4.

Fig. 3.—The effect of cobalt on incorporation of Fe^{59} into hemoglobin before and after polycythemia is produced by the cobalt administration. Differences between the incorporation before and the incorporation after polycythemia has developed are not apparent, and there is some overlap of one standard deviation between the normal and the polycythemic rats.

Fig. 4.—Comparison of Fe^{59} incorporation into hemoglobin in hypophysectomized rats with and without cobalt administration. Data on a *single* rat in which both hypophysectomy and splenectomy were done are also shown.

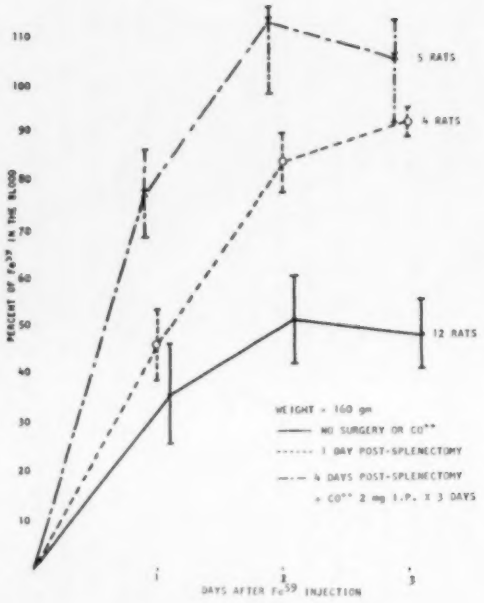

Fig. 5.

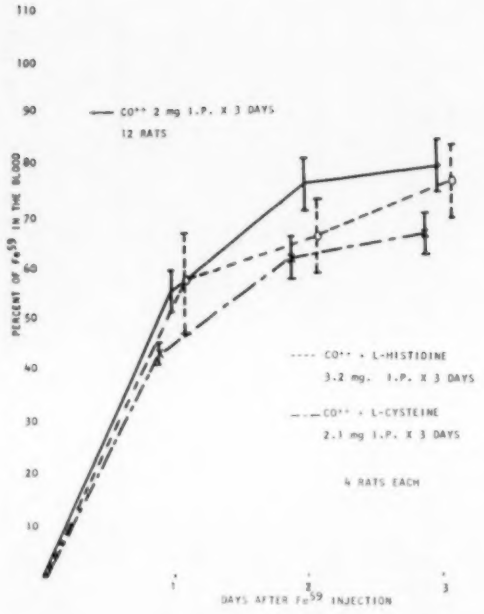

Fig. 6.

Fig. 5.—Comparison of Fe^{59} incorporation in normal and splenectomized rats and in splenectomized rats treated with cobalt. (The standard deviation of the top curve is the same above as below the mean.)

Fig. 6.—All groups of rats shown have received 2 mg. of elemental cobalt per kilogram of body weight. Each group is above the normal Fe^{59} uptake, and there appears to be questionable influence of the amino acids.

The administration of cysteine and histidine has been said to inhibit the production of polycythemia induced by cobalt in animals.[13] Solutions were prepared containing L-histidine or L-cysteine in concentrations that would permit the administration of 2 mols of the amino acid to 1 mol of the cobalt. These preparations were injected in the same syringe with the cobalt on each of the 3 days and were mixed in the syringe just prior to injection. The results are shown in Fig. 6, and suggest that there may be less effect on the iron turnover than in those animals which received cobalt alone. However, these values are not well separated and probably are not significantly different. All 3 groups of rats appeared to have a generally greater uptake than the normal groups.

DISCUSSION

The iron turnover in the rat can be measured in a 1- to 2-day period and appears to reflect changes in erythropoiesis following administration of a test material. The iron R.B.C. incorporation in rats is increased by giving cobalt 3 or 25 days. The effect of cobalt on hypophysectomized animals was found to be similar to that reported by Crafts.[15] It appears that to obtain significant results, groups of 4 or more medium-sized rats must be used, and that the weights must be uniform between the groups. Minimizing of blood loss is highly important, as the small amount of hemorrhage associated with splenectomy considerably increased the iron utilization. It is essential that the ferrous Fe^{59} citrate be injected intravenously, as it must enter the plasma pool of iron. Our experience confirms the observations of others that there is a more constant iron turnover in the hypophysectomized than in the normal animal, and that there is a proportionately greater response to stimulation of erythropoiesis.[8, 9] It is important to use iron of high specific activity so that the dose of iron may be small enough not to exceed the binding capacity of the rat plasma. A simplified formula is presented which may be applied to each animal in the group, with calculation of the mean percentage of uptake for the group. By this means, some errors in small differences in weight and hemoglobin may be avoided. When the hemoglobin values were out of line with those of the group as a whole, the data were discarded. Since full-grown female rats had 90 to 100 per cent iron uptakes in 3 days, rats were not used if their weights were above 200 grams.

SUMMARY

1. The ferrokinetics of the rat provide a reliable laboratory method of studying the effects on the rate of erythropoiesis and bone marrow function.

2. Cobalt administration increases radioiron utilization in normal and hypophysectomized rats. The action of cobalt does not appear to be altered by splenectomy or significantly modified by administration of cysteine or histidine.

3. The standard deviation of the mean results is less in the hypophysectomized group than in the normal group, and the proportional response to erythropoietic stimulation as measured by iron tracer utilization is greater in the hypophysectomized group than in the normal group.

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ASSAY OF ERYTHROPOIETIC FACTOR(S) USING RADIOIRON UPTAKE IN THE NITROGEN MUSTARD TREATED RAT

DONALD R. KORST, M.D., RONALD C. BISHOP, M.D., AND FRANK H. BETHELL, M.D.
ANN ARBOR, MICH.

A METHOD of measuring erythrocyte incorporation of radioiron (ferrous Fe^{59} citrate) in rats over a 3-day period has been described.[1, 2] The present study was designed to observe the effects of nitrogen mustard, injected intravenously, on the iron uptake by rat erythrocytes, as well as the influence on iron uptake of rats so conditioned, rats exerted by the administration of cobalt chloride, plasma and plasma extracts from phenylhydrazine anemic rabbits or bled rabbits, or plasma from patients with polycythemia or other blood dyscrasias.

MATERIALS AND METHODS

The general method of Fe^{59} detection and determination of percentage of labeled iron utilization in the rat circulating hemoglobin is essentially the same as that previously reported.[1, 2] There have been some changes in the time schedule to allow a convenient work schedule during the 72-hour period of the test. This scheduling is shown in Fig. 1, with the time of the test lasting from the initial injection of nitrogen mustard* to the last blood sample taken at the seventy-second hour, that is, 42 hours after the injection of the radioactive iron tracer. This time schedule allows an ample period for the nitrogen mustard to take full effect on erythropoiesis and yet does not permit recovery of marrow from drug suppression when the iron turnover becomes accelerated. Three blood specimens are taken at 18, 24, and 42 hours to obtain an uptake curve. Groups of white Sprague-Dawley rats in the weight range of 140 to 180 grams are used in groups of 4. The data are expressed as the mean of each group of 4, plus or minus one standard deviation represented by vertical lines. Saline or Ringer's solution is given to 4 animals with each experiment as the control group. Results are either graphed as the per cent uptake of the injected Fe^{59} in the blood or are expressed as a per cent increase over the control group. The injections of the test plasma are given either intraperitoneally or subcutaneously. The subcutaneous route has proved to be easier and more practical. Varying the periods of time between the nitrogen mustard and Fe^{59} injections was tried and different doses of nitrogen were also given to determine the optimum combination. The effects of x-radiation were also studied to determine the similarity between the iron turnovers with this procedure and with nitrogen mustard. A plastic cage, 30 cm. in diameter, was divided into wedge-shaped sections so that 8 rats plus one Victoreen radiation chamber could be exposed at the same time. X-radiation was given from a 250 K.V. unit at 80 cm. with a half-value layer of 2.4 mm. copper. The radiation used was 150 r delivered in 13.5 minutes.

From the Radioisotope Service, Veterans Administration Hospital, Thomas Henry Simpson Memorial Institute for Medical Research, and the Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, Mich.

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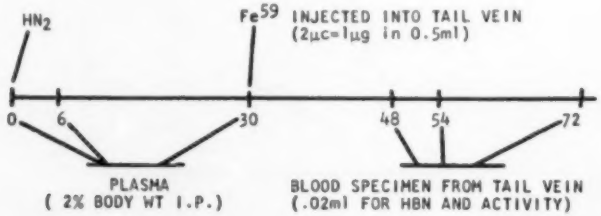
*Mustargen; Merck Sharp & Dohme (Division of Merck & Company), Philadelphia, Pa.

One milliliter of a 2½ per cent solution of phenylhydrazine hydrochloride was given to the rabbits, which weighed 6 to 10 pounds each. The material is injected subcutaneously each day for 3 or 4 days, and on the fourth or fifth day a double dose is injected. Twenty-four hours following the last injection of phenylhydrazine, hemoglobin and reticulocyte values are obtained. Rabbits are sacrificed if the hemoglobin is below 5 Gm.; if between 5 and 8 Gm., a single dose of phenylhydrazine is given, and the animals are sacrificed on the following day; if above 8 Gm., 2 additional doses are given, and the rabbits are killed on the day after the last injection. Rabbits are exsanguinated, and the citrated plasma is pooled. More recently, the syringes have been wetted with heparin solution before removal of the blood. Plasma obtained in this way and kept frozen in the whole state when not in use is designated as P.A.P. Boiled P.A.P. is similarly obtained whole plasma acidified to pH 5.5 and boiled for varying amounts of time in a water bath immediately after withdrawal (Table II). The preparation of boiled P.A.P. used in most of these studies was one that had been boiled for 10 minutes. After boiling, the protein sediment was washed with saline, and the washings were added to the supernate to bring the extract back to original plasma volume. Cresyl blue blood films on the rabbits at the end of phenylhydrazine injections usually showed 30 to 80 per cent reticulocytes with many disintegrating cells and much cellular debris, which gave the blood films a smudged appearance.



A THREE DAY ASSAY METHOD

Fig. 1.—Red blood cell incorporation of radioactive iron (Fe^{59}) in the rat as a measure of stimulation by plasma erythropoietic factor, after marrow depression by nitrogen mustard (HN_2 0.4 to 0.6 mg. per kilogram of body weight, intravenously).

Bled rabbit plasma may be prepared by a single withdrawal of blood via cardiac puncture at which time two-thirds of the blood volume, estimated as 5 per cent of body weight, is removed. This usually results in a hemoglobin of approximately 7 Gm. the day after bloodletting when the animals are sacrificed. This procedure may cause sudden death of the rabbits immediately after bloodletting, particularly in older rabbits.

Reticulocyte counts are performed on the recipient rats at the end of the study or 72 hours after nitrogen mustard administration. These are expressed as the percentage of reticulocytes per hundred red cells based on counts of 1,000 red blood cells.

RESULTS

1. The effects of x-radiation 48 hours prior to the injection of radioiron on the rate of erythropoiesis in the rat are shown in Fig. 2. These results are similar to those of Stohlman and associates.[3, 4] The widest difference in the Fe^{59} uptake appeared at 24 hours after the tracer was given intravenously. Whole rabbit phenylhydrazine anemic plasma injected as 2 per cent of body weight intraperitoneally on the same time schedule as shown in Fig. 1, produced an accelerated turnover of iron that almost approached the group of rats who

did not receive x-ray. Rats which were given 200 r in air by a similar method had a greater depression of erythropoiesis but of such a degree that the same phenylhydrazine anemic plasma did not produce any stimulation or acceleration of iron turnover over that of the control normal rabbit plasma animals. It remained to be learned if the same differences could be obtained by using nitrogen mustard instead of x-radiation.

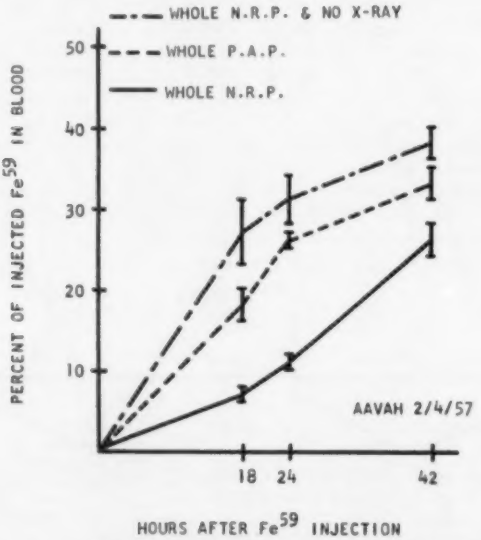


Fig. 2.—Effect of x-irradiation given 48 hours prior to Fe^{59} (total body dose: 150 r delivered in 13.5 minutes). Radioiron turnover is depressed by total body x-irradiation (————) as compared to normal animals receiving control normal rabbit plasma (- - - - - -). Plasma from phenylhydrazine anemic rabbits stimulates iron turnover in the irradiated rat (— — — —).

2. The rat (150 to 200 grams, female) erythrocyte utilization of Fe^{59} following 0.4 to 0.6 mg./Kg. of HN_2 is about 5 ± 2 per cent at 18 hours, 6 ± 3 per cent at 24 hours, and 9 ± 4 per cent at 42 hours. The normal 150 to 200 gram rat not given HN_2 has an uptake of 36 ± 10 per cent at 24 hours and 51 ± 9 per cent at 48 hours.

The effects of varying doses of nitrogen mustard on the iron turnover in the rat, and the effects of varying the time interval between the HN_2 injection and the iron tracer are shown in Table I. The largest dose tested was 0.6 mg. per kilogram of body weight given as a single intravenous dose to the rat. When no time interval was allowed between the HN_2 and the Fe^{59} injection, this dose depressed iron turnover but not to the same degree that occurred with an interval of 24 hours between the injections of HN_2 and Fe^{59}. The objection to this dose was, with an interval of 24 hours, the depression was so great that very little difference could be detected after stimulation by anemic plasma. HN_2 in a dose of 0.2 mg. per kilogram of body weight did not appear to depress

the iron uptake satisfactorily; therefore, a dose of 0.4 to 0.5 mg. per kilogram of body weight was adopted. Although an interval of 24 hours between drug and tracer injection appeared to be satisfactory, a 30-hour period fitted better into a time schedule for the entire 3-day period, and there did not appear to be any significant difference from the 24-hour interval. However, an interval of 48 hours between injections permitted a spontaneous regeneration of the bone marrow to begin before the conclusion of the uptake period. With this interval between injections only the 18-hour blood sample showed definite depression of iron utilization.

TABLE I. EFFECTS OF VARYING DOSES OF HN_2 ON THE PER CENT R.B.C. UPTAKE OF Fe^{59} IN THE RAT AND THE DIFFERENCES IN TIME INTERVAL BETWEEN HN_2 AND Fe^{59} INJECTION

DOSE HN_2 MG./K	TIME INTERVAL	PER CENT UPTAKE (MEAN OF 4 RATS ± 1 S.D.)			
		18 HOURS	24 HOURS	42 HOURS	48 HOURS
0.6	0	13 ± 3	16 ± 3	18 ± 4	
0.2	24 Hours		11 ± 5		26 ± 11
0.3	24 Hours		13 ± 1		21 ± 1
0.4	24 Hours	5 ± 2	6 ± 2	11 ± 3	
0.6	24 Hours	4 ± 1	3 ± 1	4 ± 1	
0.2	30 Hours	9 ± 1	15 ± 2	32 ± 5	
0.3	30 Hours	5 ± 2	6 ± 2	10 ± 3	
0.4	30 Hours	5 ± 2	6 ± 1	13 ± 2	
0.5	30 Hours	6 ± 2	7 ± 2	12 ± 2	
0.3	48 Hours		22 ± 4		73 ± 4
0.4	48 Hours	5 ± 2	7 ± 2	25 ± 3	

3. The effect of whole P.A.P. on iron incorporation in hemoglobin as contrasted with normal rabbit plasma (N.R.P.) is shown in Fig. 3, using the HN_2 method in the rat. It also may be noted that there was an accompanying significant difference in reticulocyte counts in these two groups of rats. The maximum percentage differences occurred at 18 and 24 hours after administration of the iron tracer, and the differences at 42 hours were less. It was our intention in this study to take 3 points of testing unknown materials in order to obtain a satisfactory curve. However, for practical purposes, it is probable that a single 18- or 24-hour specimen or both would be satisfactory. The response of the stimulated group to the control group in this study would be in the neighborhood of a threefold increase in rate of Fe^{59} utilization. When whole serum or plasma from anemic rabbits following acute blood loss 24 hours prior to harvesting the specimens was tested in this same system, it was noted that the effect was not as great as with the phenylhydrazine anemic plasma but was 180 per cent more than the control groups (Fig. 4). To eliminate the possibility that the phenylhydrazine left in the P.A.P. might in some way affect the iron turnover, 4 rats were given phenylhydrazine in the amount of 2 per cent of the daily rabbit dose which is the computed maximum amount that could remain in 2 ml. of rabbit plasma. This was given on the same schedule as test plasma for 3 injections prior to the Fe^{59} injection and did not affect the iron turnover as compared to that of control groups or normal rabbit plasma control

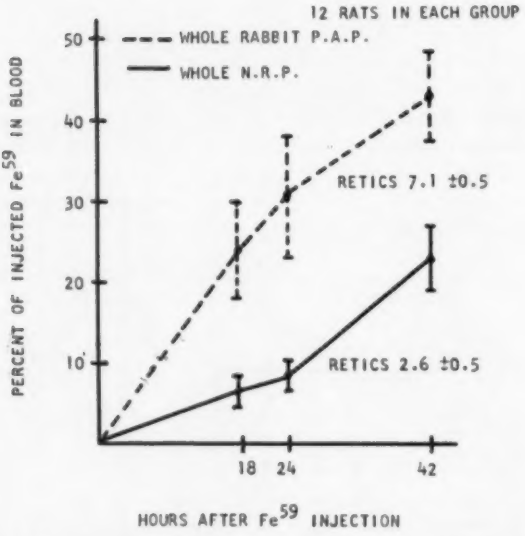


Fig. 3.—Three day erythropoietic factor assay using rats treated with nitrogen mustard. The stimulating effect of phenylhydrazine anemic plasma from rabbits (P.A.P.) is shown by a threefold or 300 per cent increase in Fe^{59} incorporation in hemoglobin of rats as well as a difference in reticulocytes.

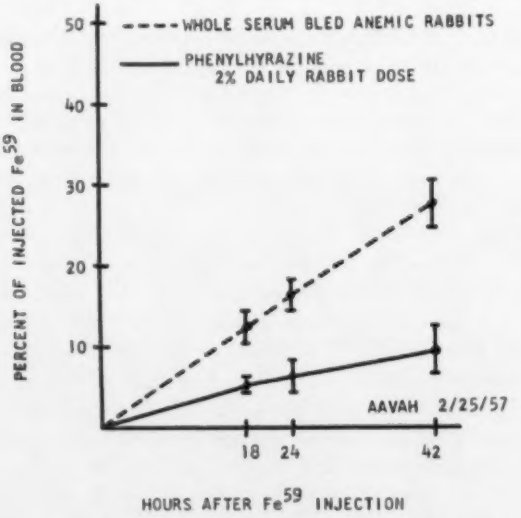


Fig. 4.—Three day erythropoietic factor assay using rats treated with nitrogen mustard. Serum from bled rabbits caused a 180 per cent increase in iron incorporation in rats (– – – –). Phenylhydrazine given directly to rats in a dose that might be present in plasma of phenylhydrazine anemic rabbits (P.A.P.) did not cause increased iron turnover (———). The Fe^{59} uptake curve of the phenylhydrazine-injected rats was identical to a group of 4 rats given normal rabbit plasma.

groups (Fig. 4). The accelerating effect of P.A.P. on the iron turnover is shown in Fig. 5 to be comparable, whether the 0.4 mg./Kg. HN_2 is given 30 hours prior to the tracer or a larger dose (0.6 mg./Kg.) of HN_2 is given at the time of the tracer injection. This would indicate that the erythropoietic activity of the P.A.P. appears to be effective to a similar degree during the time when the HN_2 is acting to depress erythropoiesis or 30 hours after HN_2 injection when it is believed that maximum depression of erythropoiesis has occurred. On the other hand, as shown in Table I, when the larger dose (0.6 mg./Kg.) of nitrogen mustard was given to rats which were not receiving erythropoiesis stimulating material, the mean uptake of radioiron at 42 hours was only 18 per cent of the administered iron in contrast to an uptake of greater than 35 per cent by untreated rats at this time interval.

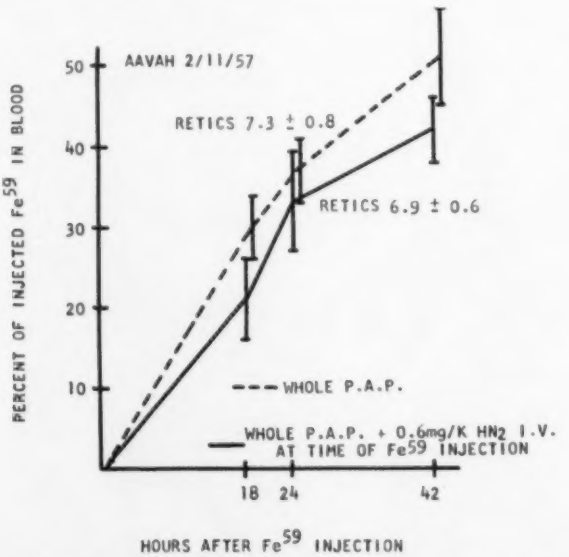


Fig. 5.—Three day erythropoietic factor assay using rats treated with nitrogen mustard. Rabbit phenylhydrazine anemic plasma (P.A.P.) increases iron incorporation in nitrogen mustard treated rats whether the tracer is given 30 hours after HN_2, 0.4 mg. per kilogram (------), or given simultaneously with a larger dose of HN_2 (0.6 mg. per kilogram).

4. In studying the erythropoietic stimulatory properties of phenylhydrazine anemic plasmas from rabbits, a number of additional observations were made. A sample of whole rabbit phenylhydrazine anemic plasma kept frozen for 9 months was found to be inactive on two separate testings by this method. A second preparation whose activity was demonstrated in Fig. 3 was kept in the whole frozen state for 5½ months in 30 ml. bottles, so that repeated thawing for testing was not a factor. This batch, tested 5 weeks after it was prepared, appeared to have retained all its potency, but when tested at the end of 5½ months was found to be only weakly active. It would appear from these

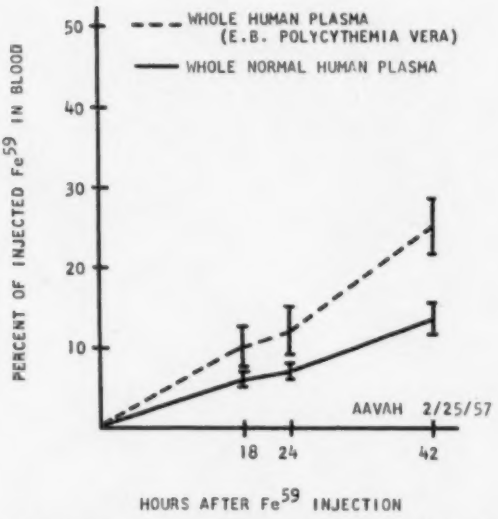


Fig. 6.—Three day erythropoietic factor assay using rats treated with nitrogen mustard. Plasma from a patient with polycythemia vera caused a 50 to 100 per cent increase in iron incorporation (-----) over that of normal plasma (———).

TABLE II. EFFECTS OF BOILING, ETHER EXTRACTION, CHLOROFORM EXTRACTION, CONCENTRATION, AND LYOPHILIZING ON ACTIVITY OF PHENYLHYDRAZINE ANEMIC RABBIT PLASMA (P.A.P.)

TEST MATERIAL	% Fe^{59} R.B.C. UPTAKE OF NITROGEN MUSTARD TREATED RATS		
	18 HOURS	24 HOURS	42 HOURS
Experiment 2/11/57			
Whole P.A.P.	30 ± 4	37 ± 4	51 ± 6
P.A.P. boiled 25 min.	7 ± 2	9 ± 2	23 ± 4
Control N.R.P.*	6 ± 2	9 ± 2	27 ± 5
Experiment 3/25/57			
Whole P.A.P.	8 ± 2	10 ± 2	20 ± 4
P.A.P. boiled 10 min.	7 ± 3	10 ± 3	23 ± 4
Ether extract of P.A.P.	5 ± 1	5 ± 1	7 ± 3
Bled rabbit plasma	7 ± 2	8 ± 2	16 ± 3
Control N.R.P.*	4 ± 2	6 ± 1	9 ± 2
Experiment 4/1/57			
Whole P.A.P.	16 ± 2	19 ± 4	34 ± 5
P.A.P. boiled 5 min.	13 ± 2	20 ± 3	35 ± 2
P.A.P. boiled 25 min.	9 ± 1	12 ± 1	23 ± 2
Chloroform extract of P.A.P.	7 ± 1	12 ± 5	25 ± 7
Chloroform residue of P.A.P.	10 ± 3	13 ± 3	24 ± 4
Control Ringer's solution	8 ± 1	5 ± 2	6 ± 2
Experiment 4/22/57			
P.A.P. conc. 5 × and lyophilized	19 ± 4	27 ± 4	42 ± 3
Bled rabbit plasma conc. 5 × and lyophilized	13 ± 3	17 ± 3	25 ± 4
N.R.P. conc. 5 × and lyophilized	9 ± 2	11 ± 3	18 ± 4
Ringer's solution	7 ± 2	10 ± 3	17 ± 3
	Mean of 4 rats ± 1 S.D.		

*Normal rabbit plasma.

observations that the erythropoietic activity of whole plasma responsible for augmentation of iron turnover had decreased on storage. The preparations whose activities are demonstrated in Figs. 3 and 4 as P.A.P. and bled anemic rabbit plasma were boiled for 5 minutes in a water bath at pH 5.5, filtered, and the filtrates lyophilized and then tested after reconstituting to original plasma volume in saline (Table II). The increases in the Fe^{59} uptake and the reticulocyte responses were of comparable magnitude to those obtained with whole plasma as shown in Figs. 3 and 4.

5. A second observation is demonstrated in Table II, in which it is noted that the longer boiling resulted in decrease of erythropoietic activity. In one experiment, P.A.P. boiled 25 minutes was little different from the control normal rabbit plasma. It would appear, under the conditions of these experiments, that the activity responsible for enhanced Fe^{59} uptake could be destroyed by boiling; therefore, our preparations are not heated for longer than 5 to 10 minutes in a boiling water bath. The material should then be placed immediately in a cold bath to prevent further heat action.

6. When a solution of $CoCl_2$ was given in a strength of 0.193 mg./Kg. or 0.386 mg./Kg. as elemental cobalt on the same test schedule as shown for plasma, there was approximately 18 per cent more red cell uptake of Fe^{59} at 24 hours and at 42 hours over the control. This is not the magnitude of response seen with bled rabbit plasma which showed a twofold increase, and the P.A.P. which showed a 3.5 fold increase.

7. The active P.A.P. was boiled for 5 minutes and filtered (activity is shown in Table II). A portion of the filtrate was mixed (separatory funnel) with chloroform in a ratio of two parts of plasma filtrate to one part of chloroform. Extraction by this procedure was carried out twice, and the chloroform was evaporated by a current of air. The dried residue was incompletely soluble in water so that a partial suspension was obtained. This material, as well as the aqueous fraction after chloroform extraction, was reconstituted to original volume of plasma and was tested. The results showed that the activities of both the chloroform extract and the remaining aqueous portion of the active P.A.P. extract were decreased to the same degree as that of P.A.P. boiled for 25 minutes, as shown in Table II. This would indicate that the factor responsible for augmentation of iron uptake may be soluble in chloroform, but it may be denatured to some extent by this procedure. An ether extract prepared in the same manner was found to be inactive by this technique (Table II). However, the residue was not tested in this instance.

8. A group of patients who had polycythemia, either secondary or primary, were studied. Patient E. B. with polycythemia vera was phlebotomized, and the whole citrated plasma was kept in a frozen state. This plasma, when tested in the nitrogen mustard treated rat system, caused an increased iron uptake over that of normal human plasma, as shown in Fig. 6; the degree of difference was quite similar to that of the bled rabbit plasma tested in Fig. 4. A second patient with polycythemia vera showed approximately the same activity as E. B. Three patients with secondary polycythemia, on testing of their whole plasmas, did not show any activity over that of normal human plasma.

9. Three patients with untreated pernicious anemia in relapse and one patient with acquired hemolytic anemia were tested using their whole citrated plasmas, and questionable degrees of increased activity were demonstrated in this system by increases of between 10 and 50 per cent at either 24- or 48-hour uptake times. There is some doubt as to the significance of these values, as the increases with bled rabbit plasma extract and with P.A.P. are in the range of 150 to 350 per cent. However, there is a suggestion of increased uptake with these patients' plasmas. Two patients with pernicious anemia in relapse were tested before and after therapeutic response to vitamin B_{12}. In the first patient with pernicious anemia, before treatment, whole plasma caused the same increase as the plasma of patient E. B.; that is, about 50 to 100 per cent increase over the control. Twenty days later, after response to vitamin B_{12}, this patient's whole plasma did not produce any increase in iron turnover in the HN_2-treated rat. Whole plasma from a second patient with pernicious anemia both before and after treatment (a span of 27 days) revealed about 36 per cent increase above control values obtained with whole plasma from normal subjects. Whole plasmas obtained from a patient with hemochromatosis and from one with aplastic anemia showed no activity above the controls.

DISCUSSION

The technique of using a nitrogen mustard conditioned rat for radioiron turnover studies has been previously reported by us.[5, 6] This type of assay has certain advantages in that normal animals are used, but it has the disadvantages of introducing variables which might modify the results. From this study, it would appear that strongly erythropoietically active substances such as P.A.P. or bled rabbit plasma can be adequately tested by this method; but it is possible that some patient plasmas may have activity too low to be detected by this procedure. The percentage differences in the iron uptake over that of the controls is probably not of the same magnitude as that observed in hypophysectomized animals.[7, 8] However, the HN_2-treated rat does offer another test system for erythropoietic factors and appears to be fairly uniform. The iron turnover rate in the rat would, moreover, probably afford an adequate parameter for measuring the erythropoietic depressing properties of various nitrogen mustard compounds. Three experiments, in which the same normal rabbit plasma and P.A.P. were used in separate groups of rats, demonstrated reproducibility of results with less than 2 per cent variation in the red cell iron uptake with either normal rabbit plasma or P.A.P. at 18 and 24 hours, and less than 6 per cent variation at 42 hours after injection of the tracer.

In general, the P.A.P. was the most potent accelerator of the iron turnover, and bled rabbit plasma or plasma extract was less potent but significantly more active than the control materials. Administration of cobalt chloride accelerated iron turnover but not to the same degree as the bled rabbit plasma or the P.A.P. A group of animals who received $CoCl_2$ in doses of 5 mg. per kilogram as elemental cobalt daily for 7 days prior to the injection of the tracer revealed an iron uptake between that of the saline control and the bled rabbit

plasma. In x-irradiated or nitrogen mustard conditioned rats, the P.A.P. increased the iron turnover almost to the level of the iron turnover in the normal rat.

Human boiled plasma extracts from patients with polycythemia have been shown to be capable of stimulating erythropoiesis in the normal rat[9] and in the hypophysectomized rat.[10] In the HN_2 test rats, several of the plasmas from patients with polycythemia vera did show activity, and in one patient the effects were almost as great as those obtained with bled rabbit plasma. But, generally speaking, the system may not have been sensitive enough to consistently detect enhanced activities of other plasmas tested. Another possibility is that the amounts of whole plasma used in these experiments, given as 3 injections (each 2 per cent of body weight), may not have been sufficient to elicit the reticulocyte changes or the increase in erythrocytic values or the total red cell mass that might be observed after 1 or 2 weeks of daily injections. It also seems probable that there may exist in plasma more than one erythropoietic factor. It has been demonstrated by means of this test system that the factor responsible for enhanced Fe^{59} uptake is not extractable by ether, is inactivated by storage for a long period, and is destroyed by prolonged heating. In a recent report by Stohlman and Brecher,[11] it was shown that the erythropoietic activity of their material was relatively thermolabile.

The factor capable of modifying iron turnover is thermolabile and present only in relatively fresh plasma. Another factor may be heat stable, ether soluble, and present in boiled plasma extracts. It increases the rate of normoblast division. The first factor is probably a protein, and the latter may be a lipid.[12]

SUMMARY

1. A new method is described to measure erythropoietic active materials utilizing the radioiron turnover in the rat treated with nitrogen mustard.

2. These studies would indicate that the erythropoietic factor which enhances Fe^{59} utilization can be destroyed on prolonged boiling, is ether insoluble, and may deteriorate on prolonged storage as frozen whole plasma.

3. Erythropoietic active plasmas or plasma extracts obtained from phenylhydrazine-treated rabbits are consistently more active than bled rabbit plasma extracts.

4. Several patients with polycythemia vera had plasmas that were active in this system.

Technical assistance was provided by Mrs. Elizabeth Ong and Mr. Peter Thoms.

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STUDIES ON ERYTHROPOIESIS

VIII. The Effect of Nephrectomy on Response to Hypoxic Anoxia

Eugene Goldwasser, Ph.D., Walter Fried, M.D.,* and Leon O. Jacobson, M.D.
Chicago, Ill.

THE existence of one or more humoral factors involved in the regulation of erythropoiesis has become accepted quite widely, and that factor which is formed in the animal in response to an anemic stress has been termed hemopoietin[1] or erythropoietin.[2] The site of production of erythropoietin is still not established unequivocally. Gordon and his co-workers[3] have demonstrated that extracts of a variety of organs from anemic animals are devoid of erythropoietic activity. Previously, it had been shown that hematopoietic tissues (radio- and nitrogen mustard-sensitive) were not involved in elaboration of erythropoietin.[4-6] Hypophysectomized animals have also been shown to respond to hemorrhage[7-9] ruling out the pituitary as a source of the plasma factor. We have shown that removal of the kidneys of experimental animals was followed by loss of the capacity to respond to hemorrhage or to cobaltous ion, by an increase in circulating erythropoietin while removal of other organs had no such effect.[10, 11] Exactly proper controls for experiments such as these are admittedly quite difficult to obtain, but in controls which were made comparably uremic by tying off both ureters, there was impairment of response only after a relatively long time, while at short times there was no inhibition of production of erythropoietin. Because of these findings we suggested that the kidney is involved in production of erythropoietin.

Recently, a paper[12] appeared in which nephrectomized animals were reported to respond to hypoxic anoxia about as well as intact animals, and the suggestion was made that there might be different sources of different erythropoietins, depending on the nature of the stimulus. In this paper we will demonstrate that the situation regarding the role of the kidney is the same whether the stress is hemorrhage or cobalt, as previously reported, or hypoxia at reduced pressure, or hypoxia at atmospheric pressure.

METHODS

Male, Sprague-Dawley rats (300 to 350 grams) were used as donors of plasma. Bilateral nephrectomy and bilateral ureter ligation were performed via a single abdominal incision. The ureters were ligated within 1 cm. of the renal pelvis and cut distally. At various intervals following operation, the rats were placed into a hypoxic atmosphere for varying lengths of time. Hypoxic anoxia was induced by exposing the rats to either a simulated altitude of 21,500 feet in a low-pressure chamber, or to an environment containing 9 per

From the Argonne Cancer Research Hospital, U. S. Atomic Energy Commission, and the Departments of Medicine and Biochemistry, The University of Chicago, Chicago, Ill.

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*Present address: Philadelphia General Hospital, Philadelphia, Pa.

cent oxygen at atmospheric pressure at a flow rate of 510 L. per hour.* The rats were bled by cardiac puncture immediately after they were removed from the chamber. The heparinized plasma was collected, pooled, and bio-assayed by methods reported previously[8, 13] to determine erythropoietin titers. In experiments in which hypophysectomized rats were used, they were purchased from the Hormone Assay Laboratories, Chicago. The results are expressed as per cent of total radioiron injected, incorporated into red cells at 20 hours. Each figure usually represents the average of values for 5 rats.

TABLE I. EFFECT OF NEPHRECTOMY AND URETER LIGATION ON RESPONSE OF RATS TO HIGH-ALTITUDE HYPOXIA

EXPERIMENT	HOURS AT 21,500 FT. SIMULATED ALTITUDE	PER CENT Fe^{59} INCORPORATION			
		UNOPERATED	URETER-LIGATED	NEPHRECTOMIZED	NORMAL PLASMA
1	8	13.2 (3.1)	10.0 (1.4)	6.9*	4.9 (2.4)
2	8	9.2 (0.6)	5.1 (0.7)	2.5 (0.5)	2.1 (0.7)
3†	8	18.8 (1.8)	15.5 (3.1)	7.3 (1.1)	7.7 (1.7)
4	8	11.5 (1.9)	9.8 (1.9)	4.3 (1.0)	4.6 (2.0)
5	8	10.9 (1.6)	9.1 (1.5)	5.8 (1.3)	4.9 (0.2)
6	10	8.5 (1.2)	8.0 (1.3)	4.4 (0.5)	3.7 (0.2)
7	16	8.6 (2.3)	9.6 (2.5)	4.5 (2.0)	5.9 (1.6)
8	24	14.7 (2.6)	14.6 (2.7)	4.7 (1.6)	3.9 (0.6)
9	24	17.0*	11.0 (2.2)	3.6 (0.9)	3.8 (0.9)

Plasma from unoperated, ureter-ligated, and nephrectomized animals obtained from rats after removal from high-altitude chamber; normal plasma is from unstimulated rats. Assays were performed in starved rats, and a total of 5 per assay group was used. Figures in parentheses are standard error of the mean.

*Three rats per group.

†Assay rats in experiment 3 had fasted one day less than usual procedure.

RESULTS AND DISCUSSION

Because of the discrepancy between our first findings with hypoxia and those in the literature,[12] we repeated the same experiment, with only minor modifications, 8 times. The results of these experiments are summarized in Table I. In each, the nephrectomized rats subjected to a simulated altitude of 21,500 feet did not produce a significant plasma erythropoietin titer. The plasma erythropoietin titer from these animals was as low as that from control rats not placed in the chamber. This was true whether the time of exposure to the high altitude was 8, 10, 16, or 24 hours. In every experiment except No. 2, the rats whose ureters were ligated before stimulation produced amounts of erythropoietin similar to those produced by unoperated rats in response to the high altitude anoxia. The degree of uremia in the ureter-ligated rats, as determined by blood urea nitrogen at each interval, was comparable to that in the nephrectomized rats. If the iron incorporation data are calculated as per cent of that given by plasma from unoperated stimulated animals and the whole series of experiments are averaged, the results may be expressed as follows: unoperated, 100; normal plasma, 38; ureter-ligated, 81; and nephrectomized, 41.

These results demonstrate quite clearly that the nephrectomized rat loses its capacity to increase erythropoietin in response to hypoxic anoxia as well as to hemorrhage or cobalt.

*We are greatly indebted to Dr. John Doull for his help with, and the use of, the low-pressure chamber and to Dr. Eric Simmons for help in construction of the low-oxygen chamber.

In order to study further the effect of uremia on the capacity of rats to produce erythropoietin in response to a low O_2 atmosphere, we performed bilateral nephrectomy and ureter ligation in groups of rats immediately prior to, 4 hours before, and 16 hours before placing them in the high-altitude chamber. In all cases, nephrectomy again abolished response to the stimulus. In the animals with ureters tied off immediately before and 4 hours before stimulating for 16 hours, the response was of about the same magnitude as that shown by unoperated animals. If, however, the ureters were tied off 16 hours before stimulus, the response was diminished but still appreciable (Table II). It seems to us that this reduction in responsiveness of the animals with ligated ureters is quite likely due to mechanical injury to the kidney by the pressures built up there. At autopsy the kidneys from rats in which the ureters had been tied off appear about twice the normal size. Their uncut surfaces are pale, and, on cutting perpendicular to the anteroposterior axis, the cut surfaces are blanched, especially in the cortical region.

TABLE II. EFFECT OF UREMIA ON RESPONSE TO HIGH-ALTITUDE HYPOXIA

HOURS BETWEEN OPERATION AND START OF STIMULUS	PER CENT Fe^{59} INCORPORATION							
	UNOPERATED	B.U.N.	URETER-LIGATED	B.U.N.	NEPHREC-TOMIZED	B.U.N.	NORMAL PLASMA	B.U.N.
0	11.6 (1.9)	24	9.8 (1.9)	57	4.3*	59	4.6 (1.1)	24
4	--	--	14.0 (0.9)	98	4.0 (1.3)	99	--	--
16	--	--	7.2 (1.9)	130	5.0 (1.0)	134	--	--

Unoperated, ureter-ligated and nephrectomized rats at a simulated altitude of 21,500 feet for 16 hours. Plasma obtained immediately after removal from chamber. Normal plasma from unstimulated rats. Assays performed in starved rats; 5 rats per assay group. Figures in parentheses are standard error of the mean. Blood urea nitrogen (B.U.N.) in milligrams per cent.

*Three rats in group.

TABLE III. COMPARISON OF LOW-PRESSURE HYPOXIA WITH ATMOSPHERIC PRESSURE HYPOXIA AND OF STARVED RAT ASSAY WITH HYPOPHYSECTOMIZED RAT ASSAY

TYPE OF HYPOXIC STIMULUS	ASSAY RAT	PER CENT Fe^{59} INCORPORATION			
		UNOPERATED	URETER-LIGATED	NEPHREC-TOMIZED	NORMAL PLASMA
Low pressure	Starved	17.0*	11.0 (1.9)	3.6 (0.4)	3.8 (0.4)
	Hypophysecto-mized	17.8 (3.5)	10.0 (0.8)	4.5 (0.2)	2.0 (0.5)
Atmospheric pressure	Starved	17.4 (1.4)	13.9 (2.0)	3.5 (0.8)	4.4 (0.5)
	Hypophysecto-mized	22.9 (8.0)	13.4 (0.8)	3.5 (1.1)	4.1 (0.9)

Low-pressure series kept at a simulated altitude of 21,500 feet (10 per cent oxygen) for 16 hours; atmospheric pressure series kept in 9 per cent oxygen for 16 hours. Plasma obtained from rats immediately after removal from chamber. Normal plasma from unstimulated rats. Five rats per assay group. Figures in parentheses are standard error of the mean.

*Three rats in group.

There still remained two possible explanations for the discrepancy between our results and those published previously.[12] The earlier work on hypoxic anoxia, reported by Prentice and Mirand,[12] was done at atmospheric pressure in a chamber containing a low, partial pressure of oxygen; also, the plasma was assayed in hypophysectomized rats rather than in starved rats as we had used. When we repeated our experiments using both of these conditions, we again found that nephrectomy completely abolished the response to low-oxygen tension

(Table III). We found no difference qualitatively between the response of hypophysectomized and starved rats used as assay animals and no difference between low-pressure hypoxia and atmospheric pressure hypoxia.

These findings have been corroborated using the polycythemic mouse for assay[14] and will be the subject of a forthcoming communication.

As of this writing, we do not know why the results obtained in the two different laboratories are so divergent, but we feel, in the light of the experiments reported here, that Prentice and Mirand's suggestion of different erythropoietins being formed in response to different anoxic stimuli is untenable.

These experiments do not, of course, establish beyond doubt that the kidney produces erythropoietin. The possibility remains that the consequences of nephrectomy in some way interfere with elaboration of the hormone by another site or that plasma from nephrectomized animals contains substances which may interfere with the primary erythropoietic process. Experiments testing these possibilities are now under way.

SUMMARY

Rats subjected to hypoxic anoxia, whether at low pressure or atmospheric pressure, respond to this condition with an increase in plasma erythropoietin. Ligation of ureters for relatively short times does not interfere with this response. Nephrectomy completely abolishes this response.

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FAILURE OF ACUTE LOWERING OF SERUM OSMOLALITY TO INDUCE DIURESIS IN MILDLY DEHYDRATED NORMAL MEN

WALTER HOLLANDER, JR., M.D.,* T. FRANKLIN WILLIAMS, M.D.,* AND MAURICE B. STRAUSS, M.D.
BOSTON, MASS.

THE ingestion of a liter of water by normal man leads to a decrease in serum osmolality of about 2 per cent and is shortly followed by diuresis. Several groups of workers[1-3] have shown that this sequence of events occurs even if the initial serum osmolality has been raised by 3 to 4 per cent by means of the prior administration of strong salt solution. Under these conditions the ingestion of a liter of water, lowering the serum osmolality by only 2 per cent, does not return this value to its initial level. Nevertheless, diuresis ensues. This has led to the concept that it is not the absolute serum osmolality, but its acute lowering which conditions diuresis.[2] Were this the case, then diuresis should ensue following the ingestion of water by mildly dehydrated man, provided serum osmolality was appropriately lowered. The following experiments indicate that this does not occur and that man, in this respect, resembles the dehydrated camel, which may drink a volume of water equivalent to 32 per cent of its body weight without developing a diuresis,[4, 5] as well as other animals which exhibit similar, if less extreme, behavior.[6]

METHODS

The subjects were healthy young male physicians. Of a total of 7 studies, 4 were performed on the fifth and seventh days of a late summer heat wave and 3 in early spring. The plan of the summer experiments was as follows: Each subject ate his usual breakfast and about one hour thereafter reclined in an air-conditioned room at approximately 70° F. during a 2-hour control period. Following this, each subject exercised by playing tennis in the hot sun for 1½ to 2 hours in order to produce a mild state of acute dehydration. After this he again rested in the air-conditioned room for two hours. At the end of the second rest period, each subject ingested approximately 17 ml. of water per kilogram of body weight during a 15- to 30-minute period. Voided specimens of urine were collected every hour or half hour for 1½ to 4 hours after the water load had been taken, the subject standing to void. Venous blood was withdrawn with minimal stasis in the middle of the initial resting period, at the end of the second rest period just before the water load, and again 35 to 50 minutes after the water load had been completely imbibed. Body weight was determined at appropriate times as a measure of acute changes in body fluid. Five grams of dry sucrose were eaten every 20 minutes throughout the study but no fluid was ingested prior to the administration of the water load. Smoking was not permitted.

From the Medical Service and Research Laboratory, Boston Veterans Administration Hospital, the Department of Medicine, Boston University School of Medicine, and the Department of Medicine, Tufts University School of Medicine, Boston, Mass.
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*Present address: Department of Medicine, University of North Carolina School of Medicine, Chapel Hill, N. C.

TABLE I. CHANGES IN URINARY FLOW AND CONCENTRATION, IN ENDOGENOUS CREATININE CLEARANCE, AND IN SERUM OSMOLALITY FOLLOWING EXERCISE AND WATER ADMINISTRATION

SUBJECT AND DATE	WEIGHT LOSS* (GM.)	WATER ADMINISTERED (ML.)	INITIAL SERUM OSMOLALITY (MOSM.)	CHANGE IN SERUM OSMOLALITY (MOSM.)			RATE OF URINE FLOW (ML./MIN.)				URINARY CONCENTRATION (MOSM.)				ENDOGENOUS CREATININE CLEARANCE (ML./MIN.)			
				BEFORE WATER	AFTER WATER	NET CHANGE	CONTROL PERIOD	DURING EXERCISE	REST PERIOD	MAXIMUM AFTER WATER	CONTROL PERIOD	DURING EXERCISE	REST PERIOD	AT TIME OF MAXIMUM FLOW	CONTROL PERIOD	DURING EXERCISE	REST PERIOD	AFTER WATER LOAD
W. H. 9-1-53	2,150	1,270	290	+5	−4†	+1	1.3	0.4	0.9	0.8	850	774	826	860	160	128	154	145
T. F. W. 9-1-53	2,430	1,340	287	+7	−9†	−2	1.8	0.5	0.3	1.0	484	476	480	470	131	110	132	131
L. G. R. 9-3-53	1,970	1,180	281	+6†	−3	+3	0.9	1.0	0.3	1.0	900	1,108	1,086	672	133	53	121	146
C. P. S. 9-3-53	2,000	1,450	284	+6	−9†	−3	1.0	0.5	0.5	1.3	1,015	984	1,092	706	143	131	135	126
T. F. W. 3-26-54	1,250	1,000	288	+3	−7	−4	1.3	0.5	0.3	4.7	673	846	1,036	137	135	121	130	127
W. H. 3-26-54	1,380	1,000	284	+6	−9	−3	1.8	0.7	0.8	1.5	609	629	740	742	163	134	160	143
W. H. 4-9-54	1,790	1,090	285	+7	−9	−2	1.8	0.6	0.5	1.3	714	706	859	694	160	134	147	153
Averages	1,853	1,190	286	+5.7	−7.1	−1.4	1.4	0.6	0.5	1.8	749	789	874	611	146	116	140	139

*Prior to water administration.

†Since cryoscopic determinations for these values were not available the change has been calculated as twice the change in serum sodium concentration.

The spring studies were similar to the 4 carried out during the summer except that the exercise was milder, was taken indoors in a room at 110° to 118° F., and was continued for 2½ to 3 hours. The amount of weight lost was less than in the summer experiments. Accordingly, a smaller water load was administered. In 2 of the 3 studies one liter of water was ingested and in the third the load was administered intravenously in the form of 1,090 ml. of a 2 per cent glucose plus 2 per cent fructose solution.

Sodium and potassium were determined in serum and urine with an internal standard flame photometer. All serum determinations were done in duplicate. Serum and urine osmolality were determined cryoscopically with a Fiske osmometer, as previously described.[7] Serum and urine chloride and creatinine concentrations were measured by the usual methods employed in this laboratory.[8, 9]

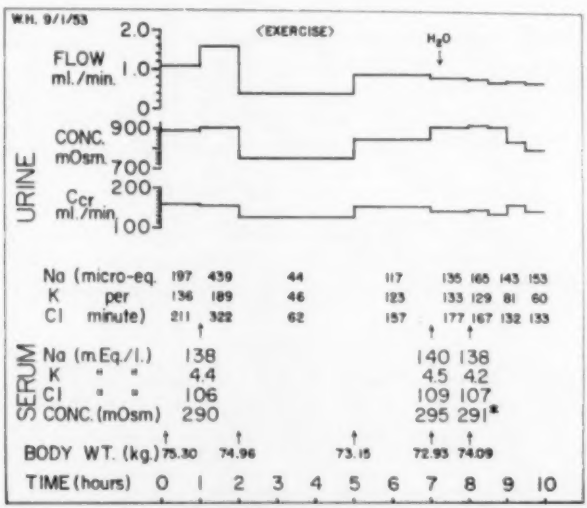

Fig. 1.—Urinary and serum changes in response to exercise and subsequent water administration.

*Calculated by subtracting twice the decrease in serum sodium concentration from the previous value for serum osmolality determined cryoscopically.

RESULTS

An illustrative example of 1 of the 4 summer experiments is depicted in Fig. 1, and of 1 of the 3 spring experiments in Fig. 2. A summary of all studies is presented in Table I. In 6 of the 7 experiments significant diuresis did not follow the administration of a liter or more of water despite the fact that this led to an average fall in serum osmolality of 7 mOsm. (a decrease of 2.5 per cent from the preceding level). In these 6 studies there was a definite, albeit slight, increase in urine flow, and in 4 there was an associated decline in urine osmolality. However, in all 6 experiments, urine osmolality remained decidedly hypertonic to that of the serum. In the remaining study (TFW 3-26-54) there was an unmistakable water diuresis with urine flow increasing to 4.7 ml. per minute and urine concentration decreasing from 1,036 to 137 mOsm. No explanation for this difference in response is at hand although it may be noted

that the amount of water ingested more nearly approximated the weight lost during exercise than in any other study, and, further, that the net decline in serum osmolality was the largest observed in any experiment.

Endogenous creatinine clearance decreased an average of 20 per cent during the period of exercise. During the subsequent rest period it rose to within an average of 5 per cent of the control value and did not change significantly after water-loading.

Although the amount of water administered was never sufficient to restore the pre-exercise body weight, the fall in serum osmolality was greater than the initial rise following exercise in 5 of the 7 studies. In part, this can probably be explained by the loss of electrolyte in sweat and urine and in small measure by the actual consumption of body tissue.

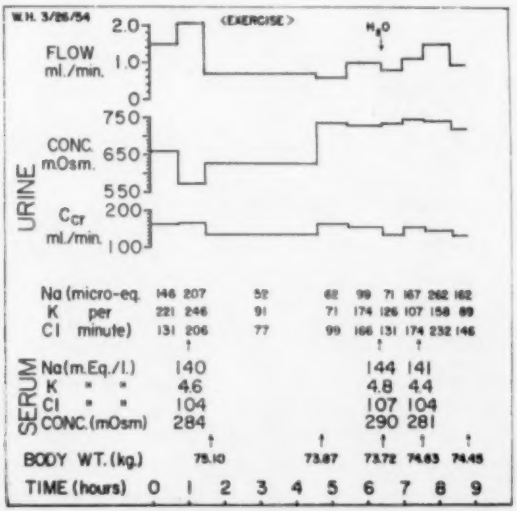

Fig. 2.—Urinary and serum changes in response to exercise and subsequent water administration.

DISCUSSION

These experiments clearly show that diuresis does not regularly follow an abrupt decline in serum osmolality averaging 2.5 per cent when the initial elevation of serum tonicity has been accomplished by the *subtraction of water* from the body. In contrast, diuresis regularly followed an even smaller decline in serum osmolality when the initial elevation had been produced by the *addition of salt* to the body.[2, 3] Certain differences are immediately apparent between these two methods of raising serum osmolality. With mild dehydration there is a roughly proportionate contraction in both intra- and extracellular fluid volumes. With strong salt administration there is a contraction of intracellular fluid volume accompanied by an expansion of extracellular fluid volume.

Certain inferences may reasonably be drawn as to the distribution of the body fluids after the ingestion of water in the two sets of experiments. In the present experiments in which sodium and chloride were lost in the urine and sweat during the course of the study and in which the volume of water administered was never sufficient to replace the total fluid lost, it is almost certain that the extracellular volume remained somewhat smaller than it was initially. In the studies involving salt administration there can be little doubt but that the extracellular fluid volume was further expanded after water ingestion. In both series of experiments the volume of intracellular fluid presumably was restored to nearly control levels since serum osmolality was so nearly returned to the initial level.

These observations, then, lend further support to the body of evidence which indicates that the volume of the body fluids, and particularly of the extracellular fluid, plays an important role in diuresis and antidiuresis.[10, 11] Mechanisms which may be involved have recently been discussed extensively[10, 11] and need not be entered upon here.

CONCLUSION

When hypertonicity of the blood has been produced by a loss of water, an abrupt lowering of serum osmolality consequent to water ingestion does not lead to diuresis as it does when hypertonicity has been produced by the addition of salt.

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THE NATURE AND DISTRIBUTION OF CARDIAC EDEMA

JOHN R. JAENIKE, M.D., AND CHRISTINE WATERHOUSE, M.D.
ROCHESTER, N. Y.

A CLEAR exposition of the abnormalities in water and electrolyte metabolism associated with congestive heart failure is essential to a further understanding of the pathogenesis of cardiac edema. Numerous investigations of this problem have been performed,[1-6] and, while general agreement exists concerning the nature of external balances during recovery from heart failure, the question of the significance of cellular abnormalities in the production of cardiac edema remains unsettled. Pertinent to this problem are the state of cellular hydration, the significance of potassium and nitrogen balance data, and the existence of cation binding and osmotic inactivation in congestive heart failure. At issue is the question as to whether the primary disturbance in body fluid metabolism is directly attributable to the renal retention of salt and water accompanying heart failure, or whether it results from a complex series of physiologic and biochemical derangements traceable to abnormal cellular metabolism.

By careful application of the metabolic balance and isotope dilution methods to the study of changes during recovery from heart failure in a heterogeneous group of patients, we have attempted to assess more completely the significance of the varying metabolic changes heretofore reported in such patients. The data indicate that the extracellular fluid space is the site of the primary disturbance in water and mineral metabolism, and suggest that the majority of observations indicating otherwise can be explained on the basis of factors which are not specific for the syndrome of congestive heart failure.

PROCEDURE AND METHODS

Subjects.—Patients with congestive heart failure manifested by gross peripheral edema, hepatic congestion, and venous hypertension were studied. Only subjects with low-output types of failure, namely those with rheumatic, arteriosclerotic, or hypertensive heart disease, are included in this report. Individual patients differed considerably in regard to the severity and duration of heart failure and responsiveness to therapy.

All patients received a low-sodium diet and digitalis, in maintenance or digitalizing doses as indicated. Certain patients received no additional diuretic therapy; others received Thiomerin intravenously or intramuscularly as indicated in the appropriate tables. Adjuvants such as ammonium chloride were given only where stipulated.

Procedure.—All studies were conducted on a metabolic balance ward, where measurements of water, sodium, potassium, chloride, and nitrogen balances were performed. Each

From the Department of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

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received a constant daily diet, which was sampled and analyzed at least twice during the course of study. Blood was drawn in the fasting state at the beginning of each metabolic period and, when indicated, more often. Daily urine collections were analyzed, and aliquots were pooled for analysis at the end of each metabolic period. Stools were separated with carmine markers and were pooled into corresponding periods. All rooms were air-conditioned, and visible perspiration did not occur. A daily skin loss of 3 mEq. of sodium and chloride was assumed.

Methods.—Sodium, potassium, chloride, and nitrogen were analyzed in diet, urines, and stools by the methods previously outlined for this laboratory.[7]

Nitrogen balances were corrected for changes in blood urea nitrogen. The value of 2.7 mEq. of potassium per gram of nitrogen was utilized in the calculation of corrected potassium balance.

Changes in extracellular fluid were calculated from chloride space, assuming the latter as 20 per cent of body weight in the edema-free state. This calculation, and those pertaining to internal cation shifts were performed by standard methods.[1]

In 6 studies on 5 subjects, body water was determined by deuterium dilution before and after diuresis. Analysis was done by the falling drop method, with minor modifications.[8] Serial blood samples were drawn up to 20 hours after administration, and complete mixing was assumed when consecutive sera showed equal or near equal concentrations. In all subjects body water balance was calculated by the method of Newburgh,[9] utilizing his modification of Lavietes' formula for the determination of fat balance. In the calculation of changes in total osmotically active cation (Table V) in subjects in whom deuterium spaces were not determined, total body water changes were back-calculated from an assumed value of 60 per cent of the nonedematous body weight. This estimated value did not enter into the calculations of body fluid balance.

TABLE I. THE RELATION OF BODY WATER LOSS TO CHLORIDE SPACE CONTRACTION IN SUBJECTS UNDERGOING DIURESIS

SUBJECT	DIURETIC THERAPY	WEIGHT LOSS (Kg.)	CHLORIDE SPACE CHANGE (LITERS)	WATER BALANCE DERIVED FROM DEUTERIUM SPACE (LITERS)		CALCULATED WATER BALANCE (LITERS)	
				TOTAL BODY	CELLS	TOTAL BODY	CELLS
I. M.	0	20.18	−16.94	−19.32	−2.38	−19.26	−2.32
J. G.	0	5.52	−4.40	−3.53	+0.87	−4.47	−0.07
W. S.	0	9.03	−6.11	−7.03	−0.92	−5.87	+0.24
Mean of spontaneous diuresis group		11.58	−9.15	−9.96	−0.81	−9.87	−0.72
H. A.	Hg*	16.13	−14.54	−15.21	−0.67	−14.16	+0.38
I. M.	Hg	6.57	−6.76	−6.44	+0.32	−6.28	+0.48
E. D.	Hg	8.41	−5.37	−5.61	−0.24	−4.93	+0.44
Mean of mercurial-treated group		10.37	−8.89	−9.09	−0.20	−8.46	+0.43
Over-all mean		10.97	−9.02	−9.52	−0.50	−9.16	−0.14
Other subjects							
C. M.	Hg	5.12	−4.76	Deuterium spaces		−4.35	+0.41
M. P.	Hg + NH_4Cl	5.62	−4.13	not determined		−4.98	−0.85
M. F.	Hg + NH_4Cl	1.91	−1.71			−1.79	−0.08

*Thiomerin given intravenously or intramuscularly.

RESULTS

A. Water Metabolism.—As shown in Table I, there was excellent agreement between changes in body water calculated from balance data and those determined directly from deuterium dilution. In all cases studied, the chloride

space contraction approximated total water loss, indicating an insignificant change in the cellular water compartment during diuresis. In the few subjects in whom some apparent loss in cell water occurred, this constituted a very small percentage of the total body water loss.

The means of production of diuresis did not appear to influence the pattern of water loss in this study. In this respect then, mercurial diuresis resembles that induced by rest, digitalis, and sodium restriction alone.

Since calculated body water changes are derived from the insensible weight loss, the question of changes in insensible loss during periods of diuresis has been investigated. Calculations were made in all patients studied in our laboratory who had undergone mercurial diuresis, and in whom control data during positive water balance or equilibrium are available. Insensible loss during periods of fluid loss was compared to that observed in control periods, and, as shown in Table II, no change was observed during diuresis.

TABLE II. AVERAGE DAILY INSENSIBLE WEIGHT LOSS DURING MERCURIAL DIURESIS, RELATED TO CONTROL PERIODS

		CONTROL		DIURESIS	
SUBJECT	DIAGNOSIS	DAYS	INSENSIBLE LOSS (GM./DAY)	DAYS	INSENSIBLE LOSS (GM./DAY)
C. M.	CHF*	13	1,283	7	1,202
M. P.	CHF	20	1,054	5	947
E. D.	CHF	11	890	16	883
H. F.	CHF	16	1,108	6	1,150
E. B.	CHF	8	909	2	785
I. I.	CHF	14	843	3	744
B. C.	Cirrhosis	19	1,277	5	1,309
J. S.	Normal	21	1,225	13	1,418
		Mean	1,101		1,105

*Congestive heart failure.

B. External Electrolyte Balances During Diuresis.—Considerable variation in the pattern of electrolyte balance during diuresis was observed, as shown in Table III. A definite grouping of subjects is apparent, however, when the sodium and chloride balances are related. This is expressed as the Na/Cl ratio in the table. In subjects showing spontaneous diuresis this ratio exceeded unity, except in Period I of I. M., when a metabolic acidosis was corrected by a chloride diuresis, and Period II of W. S., when a rather large negative potassium balance occurred for reasons which were inapparent. This pattern is simulated in Patients C. M., H. A., I. M., and M. P., who responded readily to mercurial diuretics with the loss of all gross edema. In these two groups the ratio of sodium to chloride balances closely resembled their relative extracellular fluid concentration, with the exceptions noted above.

A contrasting pattern is seen in Patients E. D., M. F., and H. F., who showed a suboptimal response to diuretic therapy, and never became entirely edema-free. Clinically, these patients had more severe and advanced heart disease, and had previously been partially or completely refractory to the conventional forms of treatment. Chloride loss exceeded sodium loss in each of these subjects, the Na/Cl balance ratio ranging from 0.44 to 0.59.

Four patients (M. P., E. D., M. F., and H. F.), including all who were relatively resistant to therapy, showed significant negative potassium balances during mercurial diuresis. In the remaining subjects, potassium balances, after correction for nitrogen, were small and variable with the exception of I. M. who retained over 100 mEq. of potassium during the correction of a metabolic acidosis. It is apparent that no consistent pattern of potassium metabolism is present throughout the entire patient group.

TABLE III. EXTERNAL ELECTROLYTE BALANCES DURING DIURESIS

SUBJECT	PERIOD	DIURETIC THERAPY	DAYS	BALANCES PER PERIOD					NaCl BALANCE RATIO	SERUM ELECTROLYTES (mEq./L.)			
				Na (mEq.)	K (mEq.)	K* (mEq.)	Cl (mEq.)	NITROGEN (GM.)		Na	K	Cl	CO_2
I. M.	I	0	7	−1,024	+137	+107	−1,140	+11	0.90	140	5.2	110	19
	II	0	7	−1,367	+56	−28	−1,164	+31	1.17	139	5.0	105	29
										140	5.0	102	29
J. G.	I	0	6	−457	+29	+27	−395	+ 1	1.16	141	3.9	100	35
	II	0	6	−276	+20	+12	−200	+ 3	1.38	138	4.9	100	34
										137	4.6	94	30
W. S.	I	0	6	−271	+7	+15	−200	− 3	1.35	134	4.6	94	30
	II	0	6	−318	−70	−56	−325	− 5	0.98	135	5.0	93	37
	III	0	7	−237	−32	0	−181	−12	1.31	137	4.8	93	35
										137	3.8	91	32
C. M.		Hg	7	−742	−41	−28	−702	− 5	1.06	139	4.7	107	27
										140	5.1	97	32
H. A.	I	Hg	5	−1,484	−78	+3	−1,423	−30	1.04	148	4.6	116	26
	II	Hg	6	−802	−46	+46	−682	−34	1.18	138	4.9	105	26
										142	5.3	101	35
I. M.	III	Hg	7	−1,016	+132	+35	−803	+36	1.27	140	5.0	102	29
										134	6.0	101	27
M. P.		Hg + NH_4Cl	6	−506	−112	−80	−421	−12	1.20	135	5.2	91	33
										135	3.9	91	33
E. D.		Hg	7	−123	−69	−58	−280	− 4	0.44	138	5.0	103	27
										137	4.3	96	30
M. F.		Hg + NH_4Cl	3	−185	−71	−71	−334	0	0.55	139	--	98	32
										139	3.9	98	31
H. F.		Hg	5	−139	−38	−22	−234	− 6	0.59	143	5.1	104	28
										137	3.3	93	37

*Corrected for nitrogen balance.

Nitrogen balances were also variable. All subjects showed some degree of chronic wasting, and the balance of nitrogen was most dependent upon the intake of calories and protein. The positive nitrogen balance in Patient I. M. accompanied an intake of 2,160 calories and 77 Gm. of protein per day and, in Patient J. G., a daily intake of 1,570 calories and 71 Gm. of protein. In contrast, the daily intake in subjects showing the largest negative nitrogen balances (W. S., and H. A., M.P., and H. F.) did not exceed 1,200 calories or 32 Gm. of protein.

C. Internal Electrolyte Shifts.—No uniform cellular cation shifts are apparent during diuresis (Table IV). In subjects showing loss of cell potassium (W. S. in Period II, M. P., M. F., and E. D.) there was a reciprocal shift of sodium into the cells. Large amounts of sodium and potassium entered the

cells during the correction of a metabolic acidosis in I. M. (Period I). In other patients, internal shifts of sodium and potassium were variable and small in magnitude and appeared unrelated to changes in cell water.

TABLE IV. INTERNAL CATION SHIFTS DURING DIURESIS

				SODIUM (mEq. PER PERIOD)			POTASSIUM (mEq. PER PERIOD)			WATER (LITERS)		
SUBJECT	PERIOD	DAYS	DIURETIC THERAPY	EXTERNAL BALANCE	Δ E.C.F.	Δ CELLS	BALANCE CORRECTED FOR N	Δ E.C.F.	Δ CELLS	CALCULATED WATER BALANCE	CHLORIDE SPACE	CELL WATER
I. M.	I	7	0	−1,024	−1,150	+126	+107	−40	+147	−8.82	−7.82	−1.00
	II	7	0	−1,367	−1,272	− 95	− 28	−52	+ 24	−10.44	−9.12	−1.32
J. G.	I	6	0	−457	−422	− 35	+ 27	−29	+ 56	−2.97	−2.63	−0.34
	II	6	0	−276	−264	− 12	+ 12	− 6	+ 18	−1.50	−1.77	+0.27
W. S.	I	6	0	−271	−208	− 63	+ 15	+ 1	+ 14	−2.25	−1.67	−0.58
	II	6	0	−318	−389	+ 71	− 56	−19	− 37	−2.44	−3.08	+0.64
	III	7	0	−237	−190	− 47	0	−23	+ 23	−1.18	−1.36	+0.18
C. M.		7	Hg	−742	−665	− 77	− 28	−19	− 9	−4.34	−4.76	+0.42
H. A.	I	5	Hg	−1,484	−1,569	+ 85	+ 3	−38	+ 41	−8.01	−9.24	+1.23
	II	6	Hg	−802	−700	−102	+ 46	−21	+ 67	−6.15	−5.30	−0.85
I. M.	III	7	Hg	−1,016	−1,067	+ 51	+ 35	−17	+ 52	−6.28	−6.76	+0.48
M. P.		6	Hg + NH_4Cl	−506	−569	+ 63	− 80	−31	− 49	−4.98	−4.13	−0.85
E. D.	I	7	Hg	−123	−197	+ 74	− 58	−18	− 40	−2.21	−1.11	−1.10
M. F.		3	Hg + NH_4Cl	−185	−243	+ 58	− 71	− 7	− 64	−1.79	−1.71	−0.08

TABLE V. THE RELATION OF CALCULATED CHANGES IN TOTAL BODY CATION TO EXTERNAL CATION BALANCE

SUBJECT	PERIOD	EXTERNAL BALANCE (mEq. Na AND K)*	CALCULATED CHANGE IN TOTAL OSMOTICALLY ACTIVE CATION (mEq.)	INTERNAL CHANGE IN OSMOTICALLY ACTIVE CATION (mEq.)
I. M.	I	−917	−1,414	−497
	II	−1,395	−1,533	−138
J. G.	I	−430	−570	−140
	II	−264	−262	+ 2
W. S.	I	−256	−285	− 29
	II	−374	−275	+ 99
	III	−237	−177	+ 60
C. M.		−770	−627	+143
H. A.	I	−1,481	−1,767	−286
	II	−756	−757	− 1
I. M.	III	−981	−1,204	−223
M. P.		−586	−735	−149
E. D.		−181	−378	−197
M. F.		−256	−272	− 16

*Corrected for nitrogen balance.

D. The Relation of Calculated to Observed Changes in Body Cation.—The relation of external sodium and potassium balances to changes in total osmotically active cation, calculated as the product of total body water and the extracellular fluid cation concentration, is shown in Table V. In the majority of subjects the calculated decrease in total cation exceeds the observed negative balance, a finding which may be interpreted as evidence of cation binding or osmotic inactivation. In relation to external electrolyte balances, this discrepancy is most striking in E. D. and Period I of I. M. Considerable variation in the direction and magnitude of this change is apparent throughout the remainder of the group.

DISCUSSION

Any group of patients suffering from chronic disease is necessarily heterogeneous. In the present study, all subjects were recovering from congestive heart failure, but differed in respect to the severity and duration of prior illness, previous state of nutrition, and food intake during the course of study. A consideration of the influence of these factors on water and electrolyte metabolism must enter into the interpretation of metabolic data derived from this type of subject, and only those metabolic changes which are relatively consistent can safely be ascribed to the common primary disease process.

The present findings indicate that the overhydration associated with cardiac failure involves largely or wholly the extracellular fluid space. This has been a consistent observation, independent of the severity of heart failure or the type of therapy utilized. The discrepancy between this finding and the conclusion reached by other workers that cell water is lost during diuresis[1-4] requires explanation. The assumption that body weight and water loss are equivalent[2-4] is erroneous unless the subject is in caloric equilibrium, and this error will reach significant proportions if caloric imbalance is large and the period of study extends beyond several days. All subjects in the present study were in negative caloric balance, most notably E. D. and W. S., in whom loss of body fat was large in relation to total weight loss. Calculations of water balance based on body weight changes in the present group of subjects would consequently result in discrepancies between chloride space contraction and total water loss which would, in certain subjects, indicate large losses of cell water.

Squires, Crosley, and Elkinton[1] have noted large discrepancies between body weight loss and urine volume during diuresis in certain cardiac subjects and have attributed this finding to excessive insensible water loss in these patients. This discrepancy has resulted in a calculated loss of water containing low concentrations of chloride, with the consequent conclusion that cellular water was excreted during diuresis. In contrast, the present data reveal no increase in insensible loss during periods of fluid loss in a variety of patients, and relating chloride and water balances reveal a chloride concentration in excess of 115 mEq. per liter of water lost in every subject but one (M. P.). The good correlation between deuterium space changes and calculated body

water changes further supports the accuracy of the metabolic method for estimating water balance. A similarly close correlation has been observed in our laboratory in patients with malignant disease.[8]

Techniques other than the metabolic balance method have been utilized in the study of cardiac edema. The ratio of exchangeable sodium to total body water in patients with heart failure suggests that the edema is primarily extracellular.[10] Similarly, muscle biopsy analyses[5, 11, 12] have generally failed to reveal evidence of cellular overhydration in the decompensated state.

The ratio of the negative balance of sodium to that of chloride during recovery from heart failure closely approximates their relative extracellular fluid concentrations in subjects undergoing spontaneous diuresis. This same relationship applies to mercurial diuresis in certain patients, namely those who appear to be readily responsive to diuretic therapy. Similar findings in both spontaneous and mercurial diuresis have been reported previously.[1, 3, 4] Barring large internal electrolyte shifts, these observations are consonant with the conclusion that the diuretic fluid in these 2 groups of patients is derived primarily from the extracellular space.

The significance of low-sodium to chloride balance ratios attended by significant potassium loss during diuresis has aroused considerable speculation in the past. That mercurial diuretics may produce these changes in some patients with heart failure has been thoroughly documented by Schwartz and Wallace,[4] and also observed by others.[5, 13] Schwartz and Wallace have demonstrated increments in ammonium and hydrogen ion excretion, as well as that of potassium, in this type of response. The identity of cations other than potassium which make up the discrepancy between sodium and chloride excretion has not been investigated in the present study. The present data are, however, compatible with the view that mercurial diuretics act to depress proximal renal tubular reabsorption of sodium chloride, and that cation exchanges subsequently occurring in the distal tubule condition the final composition of the urine. Conclusions regarding the nature of cardiac edema which are based upon analysis of the electrolyte content of diuretic fluid must take into account the mode of therapy utilized. The negative balance of potassium in this group of patients is not reflective of a primary disturbance in potassium metabolism, but is readily explainable as a phenomenon secondary to the administration of mercurial diuretics.

Patients excreting a urine with a low sodium to chloride ratio and relatively high potassium content in response to mercurial diuretics have been noted to show rapid development of mercurial unresponsiveness[4] and negligible amounts of sodium in the urine during control observations.[13] Those subjects in the present study (H. F., M. F., and E. D.) manifesting this type of response to diuretic therapy uniformly had control urinary sodium excretions under 4 mEq. per day. As previously noted, these patients had severe, advanced heart disease and were only partially responsive to intensive therapy. These findings suggest that the urinary electrolyte pattern in mercurial diuresis is related to the degree of renal sodium retention which is, in turn, influenced by the severity of the heart disease.

The loss of body water containing relatively large amounts of potassium has in the past been cited as evidence of a loss of cell water during recovery from heart failure.[4] The present data clearly indicate that water loss has occurred primarily from the extracellular fluid space, and that under certain conditions this may be accompanied by negative potassium balance and a simultaneous shift of sodium into the cells. Similar reciprocal shifts of sodium and potassium from cells to extracellular fluid have been observed by others under these conditions,[4, 13] and have been well demonstrated in experimental potassium depletion in animals[14, 15] and man.[16] While it seems probable that hydrogen ions also enter the cells in these patients,[15] the present data are insufficient to rule on this possibility.

Considerable variation in the nitrogen and potassium balances among the various subjects in this study has previously been noted. The influence of therapy on potassium balance is apparent from the foregoing, and the retention of potassium with resultant intracellular deposition, noted during the correction of acidosis in Patient I. M., indicates the influence which changes in acid-base metabolism may exert on potassium balance in this type of study. In the remaining subjects, changes in potassium balance are small, particularly in relation to total cation balance, and are best related to the dietary potassium intake. Similarly, repletion of nitrogen in these patients is most dependent upon their ability to ingest a diet sufficiently high in protein and calories to attain a positive nitrogen balance.

Electrolyte analyses of muscle biopsy specimens from patients with congestive heart failure[5, 11, 12, 17] have revealed some degree of potassium depletion in most subjects, but considerable variation of cellular sodium and potassium content has been noted. In view of the clinical differences of the subjects studied, this finding is in keeping with the conclusions derived from the present study; namely, that the presence and degree of intracellular deficits are determined by a multiplicity of factors operative in chronic disease.

The discrepancies between observed external balances and calculated changes in total body fluid cation have previously been attributed to changes in the degree of cation binding and resultant osmotic inactivation by cellular constituents.[18] Evidence of increased cellular cation binding during recovery from heart failure has been cited by several groups,[1, 2, 6] although great variability in the magnitude of this change is present within each study. This calculation is dependent upon accurate measurement of external electrolyte balances, serum concentrations, and changes in total body water, and the significance of small discrepancies must, therefore, be questioned. For example, overestimation of body water loss by one liter, probably within the experimental error in studies of this type, results in a calculated "cellular inactivation" of 150 mEq. of cation. In the present study only the results in Periods I and III in I. M., Period I in H. A., and the diuretic period in E. D. (Table V) fall outside this estimated experimental error, and all are indicative of osmotic inactivation of cation. The remaining subjects showed variable changes which were small in magnitude and of questionable significance. Aside from Period I in Patient I. M., when a shift of sodium and potassium into the cells occurred

during correction of a metabolic acidosis, no significant metabolic differences are apparent between subjects showing increased cation binding and the remainder of the group. The calculation of changes in total osmotically active cation neglects hydrogen ion balances and consequently the role of hydrogen ion as an intracellular cation, particularly in states of acidosis[19] or potassium depletion.[15] Since disturbances in acid-base balance, particularly after mercurial diuretic therapy,[4] and some degree of potassium depletion[20] are often present in cardiac failure, these factors must be considered in the interpretation of data derived from the previously discussed calculation. Similarly, although it has been hypothesized that calculated changes in cation binding occur within the body cells, the importance of bone as a cation reservoir[21] remains undefined in this and other clinical states. Evidence of changes in cation binding has been observed in a variety of subjects, both in our laboratory and elsewhere,[18] and is, therefore, not specific for heart disease. Further study is required to define the stimuli producing these changes, the role of acid-base metabolism in their production, and their site of occurrence. Until these problems are solved, it is not possible to assess accurately the true significance of internal cation binding in the syndrome of congestive heart failure.

SUMMARY

Metabolic studies have been conducted during spontaneous and mercurial-induced diuresis in a group of patients with congestive heart failure of varying severity and duration. The following conclusions are derived from the data obtained.

1. The extracellular fluid space is the primary site of fluid accumulation in cardiac edema.

2. Urinary electrolyte composition during diuresis may be influenced by therapy. Sodium and chloride are generally lost in amounts roughly proportional to their extracellular fluid concentrations in subjects who are undergoing spontaneous diuresis or who are readily and continually responsive to mercurial diuretics. The latter agents may induce negative potassium balance and low sodium to chloride balance ratios in patients with advanced cardiac disease who are partially refractory to treatment.

3. Potassium balance is also influenced by the level of dietary intake and by alterations in acid-base metabolism. Nitrogen balance is best related to the dietary intake of calories and protein.

4. The lack of characteristic changes in external nitrogen or potassium balances and the absence of consistent intracellular electrolyte shifts indicate that these alterations are not specific for congestive heart failure, but can best be explained on the basis of other factors operative in chronic disease.

5. Evidence suggesting that cation may be osmotically inactivated or bound in an unknown body site during recovery from heart failure is presented. This is an inconsistent finding, the significance of which in cardiac edema remains unknown.

6. The data favor the view that the essential primary abnormality of fluid and electrolyte metabolism in heart failure is the accumulation of excessive intravascular and interstitial fluid, secondary to the renal retention of salt and water.

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THE RELATIONSHIP BETWEEN INSULIN RESPONSIVENESS AND BLOOD GLUCOSE HALF-LIFE IN NORMAL AND DIABETIC SUBJECTS

N. Heller, M.D., N. Kalant, M.D., and M. M. Hoffman, M.D.
Montreal, Canada

IN THE assessment of patients with disordered carbohydrate metabolism, it is frequently desirable to know the response both to a carbohydrate load and to the administration of insulin. These are usually determined separately by the use of a glucose tolerance test on one occasion and an insulin or glucose-insulin test at a later date. The obvious disadvantages of such a situation have prompted the search for a procedure which would provide information in both areas simultaneously.

Recently developed methods for the assessment of glucose utilization are designed to measure the rate of disappearance of intravenously injected glucose from the circulation. Since the intravenous administration of a glucose load results in a prompt decrease in the release of glucose from the liver,[1, 2] the rate of fall of the blood sugar concentration toward normal levels is an index of the rate of glucose uptake by the tissues.[3-6] When insulin is given concurrently with the glucose, the rate of fall is increased,[7, 8] the difference in rate being a measure of the effectiveness of the exogenous insulin.

It is the purpose of this report to describe a combined test of glucose tolerance and insulin responsiveness based on measurement of the fractional rate of glucose disappearance and to determine the relationship between these parameters in normal and diabetic subjects.

MATERIALS AND METHODS

Control subjects were healthy men and women who were within the normal weight range and had no family history of diabetes in the two preceding generations. Diabetic patients had disease of the maturity-onset type beginning after the age of 40, and were free of other metabolic disturbances. Thirteen of these were not under treatment with insulin; comparison of those treated and untreated with insulin will form part of a subsequent report. All forms of long-acting insulin were discontinued 48 to 72 hours, and crystalline insulin 20 to 24 hours, prior to the test. A diet containing 250 Gm. or more of carbohydrate was taken for at least 2 days prior to the test by both the control and diabetic subjects. Experiments were performed in the morning in the fasting state with the subject recumbent. Capillary blood specimens of 0.1 ml. were obtained from the ear lobe, which was kept comfortably warm by means of an electric light bulb focused on it. A heparin-soaked sponge applied to the ear permitted repeated blood sampling from one stab wound. Glucose determinations were done by the Somogyi modification[9] of the Nelson method.[10]

From the Research Laboratory, Jewish General Hospital, and the Departments of Investigative Medicine and Medicine, McGill University, Montreal, Canada.
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Glucose was administered intravenously by injection and infusion. For the former, 35 Gm. of glucose in 50 per cent solution was given over a period of 5 minutes; for the latter, a 10 per cent solution in water was given at a rate of 72 drops per minute (controlled by a tunnel clamp) for 20 minutes. Insulin was administered intravenously; a glucagon-free preparation was diluted with commercial insulin diluent* to a concentration of 5 units per milliliter and injected from an accurately calibrated 2 ml. tuberculin syringe.

Calculations.—The rate of disappearance of glucose was calculated from the equation for a first-order reaction: $C_{(t)} = C_{(o)}e^{-Kt}$, where $C_{(t)}$ = glucose concentration at time "t," $C_{(o)}$ = glucose concentration at time zero, and K = rate constant of disappearance of regression coefficient of log glucose concentration on time. K was calculated by the method of least squares. Though others, doing similar work, have expressed their results in terms of K, it was thought that the concept of half-time is widely used and more readily appreciated; this value ($t\frac{1}{2}$) can be calculated from the equation: $K = \frac{\log_e 2}{t\frac{1}{2}} = \frac{0.693}{t\frac{1}{2}}$ which is derived from the first equation.

RESULTS

1. *Single Glucose Injection.*—In preliminary experiments, following a single injection of glucose, blood samples were obtained at 5-minute intervals for 90 minutes. The $t\frac{1}{2}$ was obtained for the 30- to 60-minute interval and for the 60- to 90-minute interval. In 8 normal subjects the mean difference in the $t\frac{1}{2}$ for the 2 periods was 1.5 ± 6 minutes ($p > 0.1$). This is in agreement with the work of others who obtained a linear relationship between glucose concentration and time for periods of 45 minutes.[5, 6]

2. *Glucose-Insulin Test.*—Fifty normal individuals and 36 diabetic patients were studied by the following procedure. An injection of 35 Gm. of glucose in 50 per cent solution was started at time zero through a 3-way stopcock attached to a needle in an antecubital vein. This was followed by an infusion of 10 per cent glucose in water at 72 drops per minute for 20 minutes and, subsequently, by saline at the rate of 10 drops per minute until the end of the experiment. Insulin was administered through a side arm of the stopcock at the sixtieth minute. The dose was 0.03 unit per kilogram for the 12 normal individuals, and 0.1 unit per kilogram for the 38 normal and 36 diabetic subjects. Capillary blood was taken at accurately timed intervals of approximately 5 minutes from the thirtieth to ninetieth minute when the procedure was terminated by the ingestion of 100 Gm. of glucose in lemon juice. If hypoglycemic symptoms developed, glucose was administered through the indwelling needle.

The half-life times were determined separately for the period prior to the administration of insulin ($t\frac{1}{2}$ G) and the period after insulin administration ($t\frac{1}{2}$ I). The former was taken as the index of glucose tolerance while the difference between them was considered as an index of insulin responsiveness. An example of the experimental data obtained is shown in Fig. 1.

Nondiabetic subjects: In nondiabetic subjects the $t\frac{1}{2}$ G ranged from 20 to 85 minutes, with a mean and standard deviation of 44 ± 18 minutes (Table I).

*We are indebted to Dr. W. R. Kirtley of Eli Lilly & Company, Indianapolis, Ind., for generous supplies of glucagon-free insulin and of insulin diluent.

This is in good agreement with the results of others using a similar procedure.[5, 11] Following the administration of 0.1 unit of insulin per kilogram, the half-life decreased to 19 ± 6 minutes, a value somewhat higher than those previously reported.[11, 12] The greater the $t_{\frac{1}{2}}$ G the greater was the decrease in half-life produced by insulin. The half-life following insulin was independent of the initial glucose half-life (regression coefficient approaching 0, Fig. 2). The results with the lower dose of insulin were almost identical, indicating that a maximal insulin effect was being obtained with both doses and that some factor other than insulin was limiting the rate of glucose disappearance and preventing a further fall in half-life. The $t_{\frac{1}{2}}$ I for the combined groups was 20 ± 6 minutes.

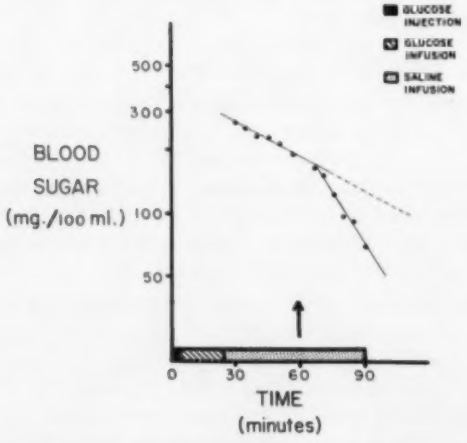

Fig. 1.—A typical normal response to the combined glucose tolerance-insulin responsiveness test. A glucose injection of 35 Gm. over a period of 5 minutes was followed by an infusion of 10 per cent glucose for 20 minutes and then by a slow infusion of saline. Insulin was given at the sixtieth minute. The glucose half-life was obtained for the periods before and after insulin.

TABLE I. INTRAVENOUS GLUCOSE TOLERANCE-INSULIN RESPONSIVENESS TEST IN NONDIABETIC AND DIABETIC SUBJECTS

GROUP	NO.	BODY WEIGHT (KG.)	BEFORE INSULIN		AFTER INSULIN	
			$K_{glucose}$	$t_{\frac{1}{2}}$ G (MIN.)	$K_{insulin}$	$t_{\frac{1}{2}}$ I (MIN.)
A. Nondiabetic 0.03 unit insulin/Kg.	12		0.0182 ± 0.0067	44 ± 18	0.0306 ± 0.0057	23 ± 4
B. Nondiabetic 0.1 unit insulin/Kg.	38		0.0183 ± 0.0070	44 ± 18	0.0417 ± 0.0170	19 ± 6
A and B	50	64.9 ± 11.3	0.0183 ± 0.0068	44 ± 18	0.0390 ± 0.0160	20 ± 6
C. Diabetic 0.1 unit insulin/Kg.	36	66.4 ± 11.7	0.0063 ± 0.0013	119 ± 35	0.0128 ± 0.0095	83 ± 50

Values shown are mean ± S.D.

The blood sugar concentration at the thirtieth minute, the beginning of the period of measurement of $t_{\frac{1}{2}}$ G, ranged from 150 to 340 mg. per 100 ml.,

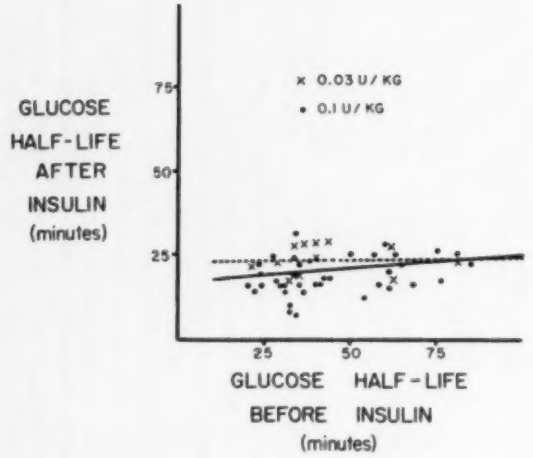

Fig. 2.

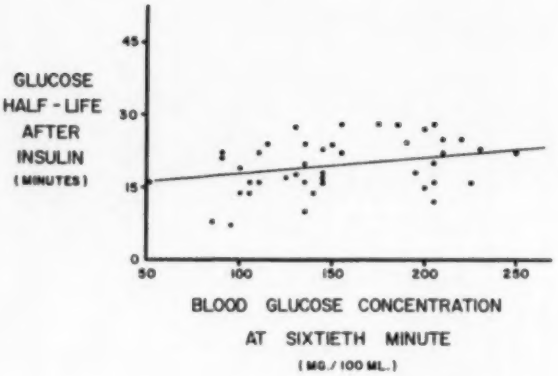

Fig. 3.

Fig. 2.—Relation of glucose half-life after insulin to that obtaining before insulin, in nondiabetic subjects. There is no significant difference in the regression lines for 2 groups of subjects receiving different amounts of insulin; for a dose of 0.03 unit per kilogram, b = 0.012 (broken line), while for a dose of 0.1 unit per kilogram, b = 0.099 (solid line).

Fig. 3.—Relationship between glucose half-life after insulin and the blood glucose concentration prior to administration of insulin. Regression coefficient b = 0.036.

varying in the same direction as the values for $t\frac{1}{2}$ G. The $t\frac{1}{2}$ I did not vary with changes in the blood sugar concentration obtaining at the time of insulin administration (regression coefficient b = 0.036, Fig. 3).

Diabetics: The $t\frac{1}{2}$ G values in diabetic subjects ranged from 60 to 225 minutes with little overlap into the normal range. Fig. 4 shows that in the diabetic group the regression line of $t\frac{1}{2}$ I on $t\frac{1}{2}$ G has a slope considerably different from that of the nondiabetic subjects and that there is much more scatter of the points about the line.

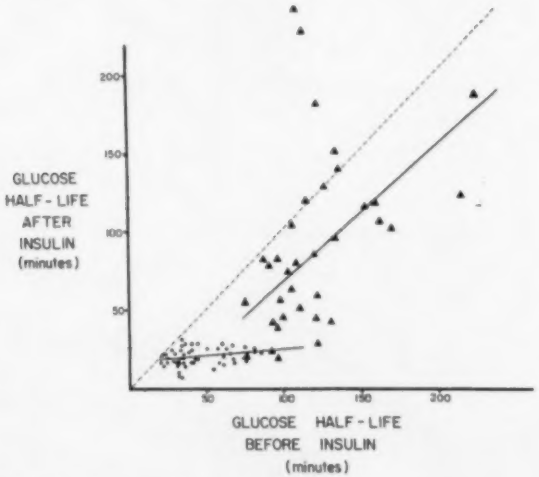

Fig. 4.—Relation of glucose half-life after insulin ($t\frac{1}{2}$ I) to glucose half-life before insulin ($t\frac{1}{2}$ G) in diabetic (▲) and nondiabetic (•) subjects. The regression lines are shown for the 2 groups, the coefficients being 0.0775 for nondiabetic individuals and 0.856 for diabetic subjects. The broken line represents the distribution for all points with complete insulin unresponsiveness ($t\frac{1}{2}$ I = $t\frac{1}{2}$ G). The vertical distance of any point from this line represents the insulin response for that subject.

DISCUSSION

The concept of the fractional disappearance rate of blood glucose from hyperglycemic levels as an index of the rate of glucose utilization has been used by several authors in recent years, but various mathematical functions of the blood glucose concentrations have been used to determine this rate.[3-6, 8, 13, 14] Our results are in agreement with those of Conard and associates[5] and of Ikkos and Luft[6] in that a linear relationship with time was obtained for the whole duration of the period of measurement only by using the logarithm of total blood glucose concentration. Recalculation of published data[3] indicated that such a relationship existed only after the fifteenth to twentieth minute following glucose injection. This has been re-emphasized by Ikkos and Luft[6] on the basis of theoretical and experimental considerations. However, by this time the blood sugar had fallen a considerable distance toward normal levels, precluding the later injection of insulin without risk of producing marked hypoglycemia. In the present work this difficulty was

obviated by the use of a glucose infusion which served to maintain hyperglycemia while equilibration was occurring, and allowing a 5-minute period for equilibration of the final portion of the infusion. The use of a 3-way stopcock facilitated rapid change from glucose to saline infusion.

The results of the first experiment, in presumptive agreement with previous reports,[5, 6, 15] indicate constancy of the disappearance of glucose over prolonged periods of time. Because of this finding it was felt that the data of the first 30-minute interval would not only permit calculation of the glucose disappearance rate, but would also act as base-line data in determining the response to insulin during the next 30-minute interval, thus enabling the measurement of both parameters during one procedure.

Normal subjects had a glucose half-life ($t_{\frac{1}{2}}$ G) ranging from 20 to 85 minutes. When insulin was administered, the half-life ($t_{\frac{1}{2}}$ I) in all subjects lay within a narrow range of 20 ± 6 minutes. This implies that the spread of values for $t_{\frac{1}{2}}$ G may have resulted from differences in the release of endogenous insulin rather than from differences in responsiveness to this insulin. The observation that, even with maximally effective doses of insulin, the half-life did not fall below a certain level (20 ± 6 minutes) indicates that some process beyond the site of action of insulin was rate-limiting, perhaps hexokinase activity.

In the present work a constant glucose load was used for subjects of different body weight, thus being, in effect, a variable load. Since Duncan[4] has presented evidence that $t_{\frac{1}{2}}$ G calculated on the basis of total blood glucose levels is dependent on the size of the load, it might be anticipated that $t_{\frac{1}{2}}$ G would now be dependent on body weight. In fact, however, these parameters were independent ($r = -0.165$, $p > 0.10$), a finding in agreement with that of others who observed no effect of load size on fractional rate of glucose disappearance.[12, 16, 17]

Previous workers have obtained values of $t_{\frac{1}{2}}$ I of 10 to 12 minutes[11, 12]; in these cases the experimental procedure differed in at least two respects—lack of a glucose infusion during the equilibration period and use of venous rather than capillary blood—so the results cannot be readily compared. It is important to note, however, that $t_{\frac{1}{2}}$ I was independent of the blood sugar obtaining at the time of insulin administration (Fig. 3).

As shown by earlier studies[3-6] the glucose half-life in diabetic subjects was considerably longer than normal. In addition, insulin did not reduce the half-life to the same level as had obtained in normal subjects. The response to insulin, indicated in Fig. 4 by the vertical distance of a point from the broken line ($t_{\frac{1}{2}}$ G = $t_{\frac{1}{2}}$ I, insulin response zero), was seen to vary continuously from normal through zero to a negative value. These results support the contention of Himsworth and Kerr[18] that patients with maturity-onset diabetes are, on the whole, relatively unresponsive to insulin. Though the urinary loss of glucose probably played an insignificant role in determining the glucose disappearance rate of normal subjects,[13] it may have been important in the diabetic patients since the blood sugar concentrations were somewhat higher.

Insulin decreases the tubular reabsorption of glucose in diabetic patients[19] and thus increases the rate of glucose disappearance; a change in $t_{\frac{1}{2}}$ induced by insulin may thus be due to changes in peripheral, hepatic, or renal glucose metabolism. The finding of decreased responsiveness to insulin, as this is defined in the present work, offers no explanation as to the mechanisms of the decrease.

In general, as the value for $t_{\frac{1}{2}}$ G increased there was an increasing discrepancy between $t_{\frac{1}{2}}$ I values of normal and diabetic subjects. There are 3 possible explanations for such a finding.

1. The maximal response to the administered insulin may have been reached when the $t_{\frac{1}{2}}$ G was about 85 minutes and the $t_{\frac{1}{2}}$ I about 20 minutes; the difference of 65 minutes was the greatest response to insulin seen in control subjects, and increases in $t_{\frac{1}{2}}$ G above 85 minutes would then be associated with equal increases in $t_{\frac{1}{2}}$ I.

2. There may have been a continuously decreasing responsiveness to insulin, both endogenous and exogenous, manifested, respectively, by an increasing $t_{\frac{1}{2}}$ G and a decreasing ability of insulin to lower the half-life to the normal value of 20 minutes.

3. There may have been a continuous increase in the "noninsulin" rate-limiting factor(s), with the same effects as those of a decreasing insulin responsiveness.

The first of these explanations is unlikely since those patients with long glucose half-life had, on the whole, smaller insulin effects than the 60-minute reduction in $t_{\frac{1}{2}}$ G shown by some of the normal individuals. At the present time there is no basis for choosing between 2 and 3 as the true explanation. However, there appears the very distinct possibility that the diabetic syndrome in some subjects may be due not to a lack of insulin or direct inhibition of insulin action on cell permeability, but to a metabolic block on the glycolytic pathway, making the step concerned rate-limiting and, secondarily, preventing a full response to insulin.

The response of any individual to a test dose of insulin may now be compared with that of other individuals having the same "base line" of glucose utilization by referral to data such as those shown in Fig. 4. A subject whose glucose half-life after insulin is greater than that anticipated from the value before insulin may be considered to show relative insulin unresponsiveness, without, however, implying any specific mechanism for such an abnormality.

SUMMARY

A method has been devised to permit the measurement of blood glucose half-life after glucose loading, before and after the administration of insulin. The former value was taken as an index of glucose tolerance and the difference between the two as an index of insulin responsiveness. In normal subjects the half-life of intravenously administered glucose ranged from 20 to 85 minutes. After the injection of insulin the half-life was 20 ± 6 minutes regardless of its

initial value. Diabetic subjects showed a decrease in glucose tolerance reflected in an increased half-life; as this abnormality increased in severity, the inability of exogenous insulin to lower the half-life to normal levels increased proportionately. This may have been the result of decrease in the specific response to insulin or of an increase in a noninsulin rate-limiting factor.

We are indebted to Dr. J. L. Wolfson for much helpful advice and criticism.

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STUDIES ON GROWTH HORMONE

IV. The Effects of Changing Amino Acid Concentrations Upon the Hormonal Regulation of Protein Metabolism in the Isolated, Perfused Liver

John B. Price, Jr., M.D.,* and John F. Prudden, M.D., Med. Sc.D.
New York, N. Y.

IN THE first of this series of studies the effects of varying hormonal environments upon the protein metabolism of the isolated, perfused rabbit liver were studied.[1] These experiments were carried out in the presence of high perfusate amino acid levels—a condition believed to be requisite to the maximal stimulation of protein synthesis. The demonstration of differences in the action of growth hormone (GH) as the amino acid level is varied in the intact animal[2] suggested similar differences might be apparent in the perfused liver. Moreover, the determination of the actions of growth hormone and insulin at amino acid levels approximating those in the intact animal seemed desirable. Accordingly, in this study the actions of GH and insulin were examined at physiologic amino acid concentrations and the results compared with those previously obtained.[1] Marked differences were demonstrable in several of the studied effects of these two hormones at these two quite different perfusate amino acid levels.

METHODS

Apparatus and Technique.—The apparatus and operative technique were those previously described[1, 3]; however, no provision was made for collection of carbon dioxide. The common bile duct was cannulated and bile collected in all experiments.

The Perfusate.—The donor blood was collected under sterile conditions by cardiac puncture and the final perfusate was composed of the following: 500 ml. of defibrinated rabbit blood, 500 ml. of an amino acid solution composed of 0.246 Gm. amino acid mixture,† and 5.36 Gm. sodium chloride; the final mixture was adjusted to pH 7.45. In addition, 4.0 mg. of glycine-2-C^{14} with a specific activity of 1.45 mc. per millimole and a radiopurity of > 99 per cent was added to the perfusate.‡ Table I gives the approximate millimolarity of the perfusate.

The Experimental Protocol.—This is unchanged from the previous experiments.[1] Hourly samples of perfusate are removed for chemical and radioactive determinations. When GH§

From the Department of Surgery, Columbia University, College of Physicians and Surgeons, New York, N. Y.

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‡Synthesized by the Isotopes Specialties Company.

§We are indebted to Dr. C. J. O'Donovan of the Armour Laboratories for the growth hormone used in this study. This growth hormone lot (Armour No. K491025) was reported to contain 0.32 ± 0.098 unit per milligram thyrotropic activity.

was to be tested, 50 mg. was added to the perfusate just prior to perfusion. Similarly 40 units of regular insulin was added in insulin experiments, and both agents in these dosages in the combination GH-insulin experiments.

TABLE I. APPROXIMATE MILLIMOLARITY OF THE PERFUSATE

PERFUSATE	MILLIMOLES/LITER
A. Amino Acids	
1. Arginine	0.035
2. Glycine	0.760
3. L-Histidine	0.067
4. Isoleucine	0.151
5. Leucine	0.310
6. Lysine	0.159
7. L-Methionine	0.067
8. Phenylalanine	0.079
9. L-Threonine	0.395
10. DL-Tryptophan	0.022
11. L-Baline	0.148
12. L-Glutamic acid	0.025
B. Other Constituents	
13. Lactic acid	0.27
14. Na^+	164.05
15. Cl^-	138.05
16. K^+	2.3
17. CO_2	1.8
	308.69

Chemical Procedures.—The red cell free "plasma" perfusate was analyzed by the following techniques: (1) urea: Gentskow and Masen[4]; (2) glucose: Nelson[5]; (3) perfusate protein: Micro-Kjeldahl, Ma and Zuazaga[6] (hemoglobin corrections on the perfusate "plasma" for lysed erythrocytes were made as before[1]); (4) alpha amino nitrogen: Albanese[7] and Fister.[8]

Radioactive Measurements.—The radioactivity of the plasma proteins and the estimation of the isotopic dose were determined as before, except that a predominantly helium mixture rather than methane was employed.

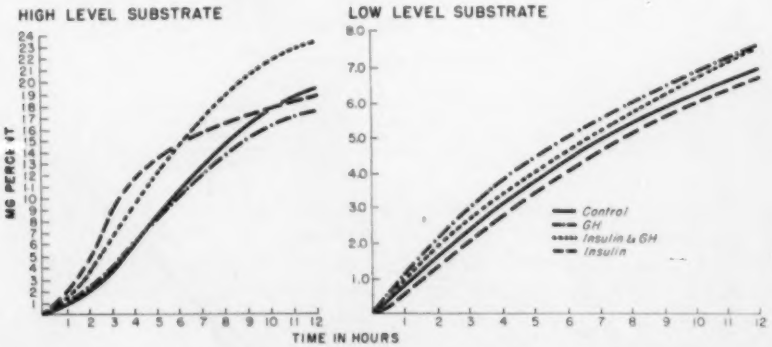

Fig. 1.—Perfusate urea nitrogen.

RESULTS

Data for the results presented in Figs. 1 to 4 were obtained in 18 perfusion studies: 6 controls, 5 GH, 4 insulin, and 3 GH-insulin. For purposes of compari-

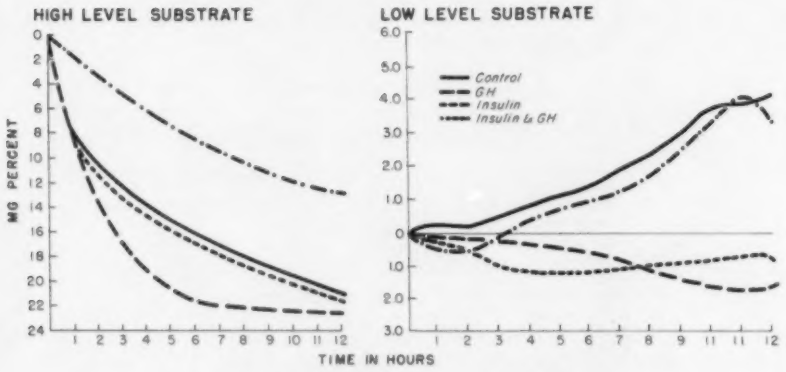

Fig. 2.—Perfusate alpha amino nitrogen.

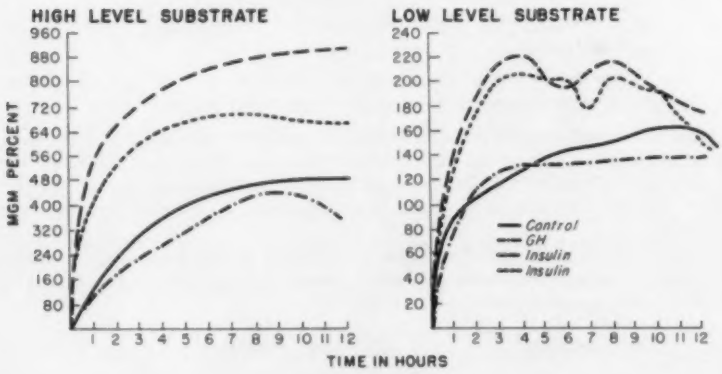

Fig. 3.—Perfusate glucose.

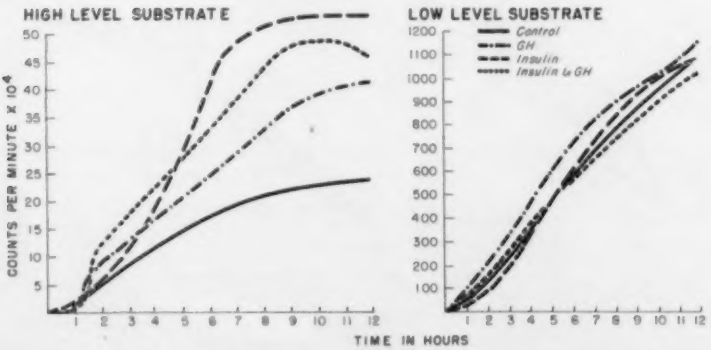

Fig. 4.—Perfusate protein radioactivity.

son, the previously obtained results from the high-level amino acid experiments* are given. Table II presents the statistical characteristics of the low-level substrate data. In each box of the table the larger member of the comparison listed is given with "p" as the probability for identity between the

TABLE II. STATISTICAL ANALYSIS OF PERFUSATE CONSTITUENTS UNDER VARYING ENDOCRINE ENVIRONMENTS*

A. LOW-LEVEL SUBSTRATE PERFUSATE UREA NITROGEN

		GH	GH-INS.	INSULIN
Control	versus	GH $p < 0.001$	GH-Ins. $p < 0.001$	Control $p < 0.005 > 0.001$
GH	versus		GH $p < 0.3 > 0.2$	GH $p < 0.001$
GH-Ins.	versus			GH-Ins. $p < 0.001$

B. LOW-LEVEL SUBSTRATE PERFUSATE ALPHA AMINO NITROGEN

		GH	GH-INS.	INSULIN
Control	versus	Control $p < 0.001$	Control $p < 0.001$	Control $p < 0.001$
GH	versus		GH $p < 0.001$	GH $p < 0.001$
GH-Ins.	versus			GH-Ins. $p < 0.5 > 0.4$

C. LOW-LEVEL SUBSTRATE PERFUSATE GLUCOSE

		GH	GH-INS.	INSULIN
Control	versus	Control $p < 0.01 > 0.005$	GH-Ins. $p < 0.001$	Insulin $p < 0.001$
GH	versus		GH-Ins. $p < 0.001$	Insulin $p < 0.001$
GH-Ins.	versus			Insulin $p < 0.05 > 0.025$

D. LOW-LEVEL SUBSTRATE PERFUSATE PROTEIN RADIOACTIVITY

		GH	GH-INS.	INSULIN
Control	versus	GH $p < 0.005 > 0.001$	Control $p < 0.02 > 0.01$	Control $p < 0.1 > 0.05$
GH	versus		GH $p < 0.001$	GH $p < 0.001$
GH-Ins.	versus			Insulin $p < 0.05 > 0.025$

*The type of experiment indicated within each box shows that one of the experimental pair being compared which exhibited the higher mean for the total 12-hour perfusion. The probability that the difference between these means was due to chance selection is given by the p value in each square.

types of perfusions compared. In all comparisons and in all data plotted, the values are "normalized" by converting the hourly samples to increments or decrements from a starting level of zero; "p" was obtained by means of a "t" test with pairing of the 12 hourly means against one another. Fig. 5 shows the mean differences between the measured perfusate constituents in the present experiments over the perfusion time from 1 to 12 hours, inclusively. This figure offers the simplest visual estimate of the over-all behavior of these constituents, but it lacks the more complete information given by hour-to-hour changes in perfusate concentrations. Certainly the most accurate comparison

*Hereafter these are referred to as "high-level substrate" and the current experiments as "low-level substrate."

would be provided by a calculation of the constants which characterize the increments or decrements of these concentrations with time. These results will be reported at a later time.[9]

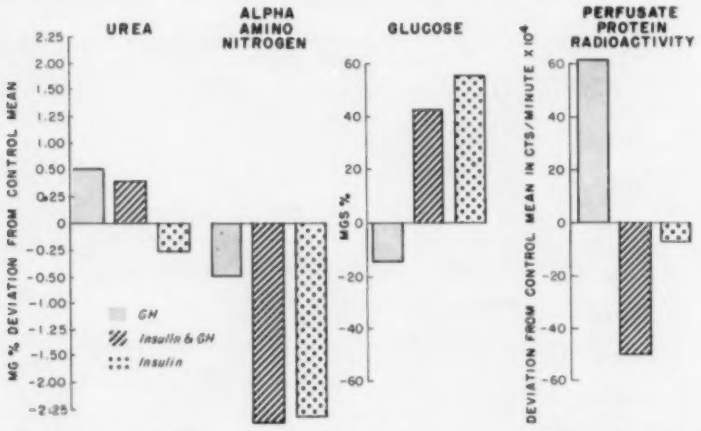

Fig. 5.—The changes induced by insulin and growth hormone in the mean 12-hour perfusate concentrations relative to the controls.

The means and standard deviations of the initial concentrations of the constituents considered are listed below:

1. Urea	11.6 ± 2.00	mg. per cent
2. Glucose	57.6 ± 4.17	mg. per cent
3. Total "plasma" protein	2.44 ± 0.22	mg. per cent
4. Alpha amino nitrogen	6.8 ± 1.31	mg. per cent

DISCUSSION

The graphic comparisons of the data obtained under the two levels of perfusate amino acid concentrations with the same hormonal environment show striking differences between the two groups.

Urea (Fig. 1).—High-level substrate GH experiments produced less urea than all other types, and the insulin and GH-insulin experiments produced more than the control. In contrast, the low-level substrate GH experiments produced more urea than the control, while the insulin perfusions produced less urea. Thus the effect of growth hormone on urea production is dependent on the amino acid level, and at low levels GH actually increases this particular metabolic index. The effects of insulin are also reversed as decreased urea production is noted in the low-level results. Data obtained in nephrectomized animals[2] treated with GH reflect these hepatic results, since GH treated animals receiving a load of amino acids show a decreased rate of urea formation; however, the rate of urea formation is little affected in a growth hormone treated animal without the amino acid load.

Alpha Amino Nitrogen (Fig. 2).—In the high-level experiments with an initial alpha amino nitrogen value of 170 mg. per cent, there was a large removal of amino acids by the liver with a resultant drop in perfusate levels in all hormonal environments including the controls. Insulin produced the largest removal by the liver, and growth hormone alone the smallest—even less than the control. At an initial level of 6.8 mg. per cent, however, there was a rising level of perfusate amino acids in GH and control experiments. The insulin perfusions (either alone or with growth hormone) exhibited an hepatic amino acid uptake with a falling perfusate amino acid concentration. In this environment the liver functions similarly to "peripheral tissues," since eviscerate animal preparations have shown increased tissue uptake of plasma amino acids with insulin.[10, 11] Insulin experiments thus show an increased uptake of amino acids that is relatively independent of perfusate amino acid levels.

An explanation in accord with the greatly decreased hepatic uptake of amino acids in the high-level substrate GH experiments is an increased production of amino acids and hepatic proteins. This would also apply to the rises observed in human plasma protein and nonprotein S^{35} when GH is given following a prior administration of S^{35}-labeled methionine.[12] The amino acid uptake data with GH and a low-level substrate show an outpouring of hepatic amino acids; however it is less than in the control experiments. As in the case of urea production, the action of GH upon perfusate alpha amino nitrogen level is seen to be dependent on perfusate amino acid levels.

Additional amino acid level-dependent differences are seen when perfusate protein radioactivities are compared. In the high-level studies, all experimental hormonal environments markedly increased the rate of C^{14} incorporation as compared to the control environment. As would be expected in a closed system, there was a tendency for all curves to plateau with time.

In the low-level experiments, although statistically significant differences are still present, the experimental environments show less deviation from the control. Insulin (with or without GH) no longer increases the rate of C^{14} incorporation, while GH (alone) still causes a higher rate of incorporation than the control.

Glucose (Fig. 3).—In both series of experiments a rising level of perfusate glucose was observed in all hormonal environments. Significantly higher levels of glucose production were present in the insulin experiments, and the lowest levels were consistently seen with GH. At both amino acid levels the addition of GH-insulin lowered glucose perfusate concentrations from their mean insulin experiment levels; but these exceeded the control level. Glucagon-free insulin* was employed at both amino acid levels, and the lack of any discernible difference from the other insulin runs clearly indicated that the hyperglycemic effects were insulin-induced. Hepatic glycogen levels were not decreased in the high-level substrate insulin experiments so that increased glycogen breakdown is not the source of the perfusate glucose. The much greater absolute production of glucose in the high-level amino acid experiments indicates the amino acid pool

*Kindly furnished by Eli Lilly & Company, Indianapolis, Ind.

is the source. Sequence of action studies of the effects of insulin on liver slices have shown[13] that increased glucose uptake is produced only by relatively long periods (12 to 18 hours) of contact with insulin, in contrast to muscle studies where an almost immediate increased glucose uptake occurs. Therefore, in the nonfasted animal the first action of insulin upon the liver results in an increased production of glucose plus an increased uptake of amino acids.

Perfusate Protein (Fig. 4).—In the high-level substrate experiments[1] a consistent difference was noted in the perfusate protein values when determined by the biuret method and by the micro-Kjeldahl technique. This difference was largest in the insulin experiments, and was considered due to a polypeptide fraction measured in the biuret reaction, but which was not precipitated by the trichloroacetic acid used preparatory to the micro-Kjeldahl procedure. This fraction has not at present been isolated or characterized. In the low-level substrate experiments this fraction could not be consistently measured, and when demonstrated it was considerably smaller than in the earlier series of experiments. Thus, although this fraction appears to be characteristic of hepatic metabolism at very high amino acid levels, it is present at physiologic amino acid concentrations. The high-substrate data previously presented[1] indicated that this fraction was composed of synthesized polypeptides, since the information obtained showed that a considerable protein synthesis was under way. The possibility certainly exists that some of this trichloroacetic acid soluble fraction was due to proteolysis of hepatic protein; however, the fact that the fraction went to low or undetectable levels in the low-substrate experiments appears to be a strong confirmation of the view that it is *not* proteolytic in origin, since the amount of hepatic protein available as a proteolytic substrate was essentially the same in both instances and since all conditions were similar except for the perfusate amino acid concentrations. If the experimental technique resulted in large-scale aggregate proteolysis, as in liver mince or liver slice preparations, the trichloroacetic acid soluble fraction would have remained at approximately the same level in both types of experiments carried out. This was clearly not the case.

In general, GH and insulin produced opposite effects on the chemical indices measured, and their combination produced intermediate results. However, as there is evidence of insulin elaboration with GH administration in the intact animal[14]; and since insulin appears necessary in most circumstances[14] for a positive nitrogen balance, some of these intermediate responses of the perfused liver may be consistent with a maximal anabolism in the intact animal. The increased peripheral uptake of amino acids and glucose which insulin is known to induce could conceivably provide the conditions necessary for maximal anabolic and anticatabolic effects of growth hormone.

SUMMARY

The effects of GH and insulin upon the metabolism of perfused rabbit liver are altered considerably by changes in the perfusate amino acid levels. Thus,

with physiologic levels of amino acids and in contrast to the "high-level" experiments:

1. GH no longer decreases urea production, but actually increases it.

2. Insulin decreases urea production.

3. GH produces a rising amino acid level, and insulin produces a falling amino acid concentration. These effects are directionally in agreement with the high-level results; however, the low-level GH curve ascends to a plateau while the high-level GH curve *descends* to its plateau.

4. Both GH and insulin-induced C^{14} perfusate protein activity were much closer to control levels than in the presence of high alpha amino nitrogen concentrations.

5. The directional effects of both hormones on perfusate glucose concentrations were independent of amino acid concentration, insulin causing hyperglycemia and GH hypoglycemia. These effects on the isolated liver are directly opposite to those seen following the administration of these hormones to intact animals.

6. The results indicate that GH increases amino acid and protein turnover rates with low-substrate levels, while decreasing them with high levels. In contrast, insulin acts to slow amino acid and protein turnover at low alpha amino acid levels, and speed it (relative to both GH and controls) at high levels. Various facets of this interesting relationship are discussed.

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STUDIES ON THE RESPIRATION OF HEART MUSCLE IN VITRO

With Special Reference to the Effect of Oxygen Concentration

Ernst Peschel, M.D., and Ruth Georgiade, M.A.
Durham, N. C.

THE manometric method of Warburg was used to measure the basal respiratory rate of heart slices. In a subsequent series of experiments, the effect of oxygen concentration on the respiratory rate was studied. The oxygen consumption of the heart slices was found to be dependent upon oxygen concentration. This dependence could be due to the sensitivity of the individual cell to a lowered oxygen concentration, or to the insufficient diffusion of the oxygen within the heart slice. A third series of experiments studied this problem.

No definite values are established for the metabolism of heart muscle as far as the basic metabolic reactions of respiration and glycolysis are concerned. The rate of respiration of normal rat heart muscle slices is supposed to vary considerably from animal to animal. Data in the literature, relatively scarce considering the importance of the subject, reveal variations of Q_{O_2}* for rat heart slices from 4.7[1] to 21.4[2] under apparently similar conditions. Average Q_{O_2} values are reported as 4.9,[1] 10.0,[3] 7.9,[4] 12.5,[5] 5.0,[6] and 12.8.[7] However, heart muscle like any other living tissue must have a basal metabolism which should be expected to have a definite level. One group of investigators reported average values for ventricle and auricle separately as 7.8 and 15.7, respectively,[2] but this difference was not found by other workers[5] nor in our preliminary experiments.

METHODS

Adult rats of Osborne-Mendel strain, which had been fed Purina Dog Chow and tap water ad libitum, were killed by a blow on the head and decapitation. The heart was removed immediately and placed in oxygenated Ringer-glucose solution modified as described below. Slices, prepared from the ventricles using a Stadie-Riggs slicer, were kept in oxygenated incubation medium until slicing was finished and immediately transferred into the Warburg vessels. Trimming and all manipulations were kept at a minimum. The total period from killing the animal until the vessels started shaking in the constant temperature bath lasted 7 minutes.

Kidney, liver, and spleen slices were handled in the same manner. Diaphragm was removed in toto from young rats weighing 30 to 50 grams; the two sides of the diaphragm were separated from the connective tissue by cutting with a scissors.

From the Department of Medicine, Duke University Medical Center, Durham, N. C.

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*Q_{O_2} = mm.³ oxygen consumed in 1 hour by 1 mg. of dry weight of tissue.

Each vessel contained 2 ml. of the modified Ringer's solution described by Levy.[5] Its composition is: Na 152.4, K 4.8, Ca 1.7, Mg 1.8, Cl 127.6, SO_4 1.8, and PO_4 31.3 mEq./L.; glucose 0.01 M; pH 7.2. Respiration was measured by the direct method of Warburg, with 0.2 ml. of 10 per cent KOH in side arm or center cup of the vessel. The gas phase was 100 per cent oxygen, mixtures of oxygen with nitrogen, or air.

After saturation with gas for 5 minutes the vessels were equilibrated for 10 minutes. The rate of shaking was 160 oscillations per minute. The readings, started 22 minutes after the death of the animal, were done at 10-minute intervals.

The approximate thickness of the slices was determined from wet weight and surface area. The slices were weighed after drying, and the wet weight was assumed as 4 times the dry weight for heart and 5 times the dry weight for the other tissues, ratios which, in preliminary measurements, were found to be very constant. The oxygen consumption was calculated on the basis of the dry weight of the tissue.

Lactic acid determinations were done according to the method of Barker and Summerson.[8] In order to estimate the lactic acid production of successive periods, slices were incubated for varying intervals of time; values for the later periods were obtained by subtracting the values found for shorter periods from those found for longer periods. It is realized, of course, that this method gives more the general trend than quantitative accuracy.

No experiment was excluded except for obvious technical deficiencies.

RESULTS

Respiratory Rate of Rat Heart Muscle Under Optimal Conditions.—Oxygen consumption was relatively constant for 2 hours. The respiratory rate calculated on the basis of the first 30-minute period was considered to represent the true resting metabolism of the heart slice and was used throughout. In 279 experiments in 100 per cent oxygen, the average basal respiratory rate of heart slices not exceeding 0.5 mm. thickness was 17.4 ± 3.0. Variations from experiment to experiment were not excessive as is apparent from the standard deviations. Multiple slices from the same heart in the same experiment showed very similar respiration rates provided the condition of the slices was comparable. Some examples are listed in Table I.

TABLE I. RESPIRATORY RATE OF RAT HEART MUSCLE: COMPARISON OF Q_{O_2} OF SEVERAL SLICES PREPARED FROM THE SAME HEART
(Measured in 100 per cent oxygen)

SLICE:	1	2	3	4	5	6
Rat 1	17.8	17.5	18.4	16.4	18.4	17.4
Rat 2	15.9	14.4	14.3	17.2	15.7	15.2
Rat 3	17.0	17.6	16.4	17.8	16.6	16.8

Effect of Oxygen Concentration on Respiratory Rate of Heart Muscle.—If the respiration of heart muscle slices is measured in air instead of pure oxygen, the rate is considerably lower. In 233 experiments, the average basal respiratory rate of heart slices not exceeding 0.5 mm. thickness was 9.9 ± 1.9.

Average Q_{O_2} values for heart slices at various oxygen concentrations are listed in Table II. It is apparent that the respiratory rate is constant between 100 and 60 per cent oxygen, and decreases progressively from 50 per cent downward. In 10 and 5 per cent O_2, average Q_{O_2} values of 6.1 and 2.6, respectively, were found. However, in order to secure adequate diffusion, these low

oxygen concentrations require such extremely thin slices that the condition of the slices is inadequate. The slice technique was, therefore, considered to be unsuitable for studies of the effects of oxygen concentrations lower than that in air, and such experiments have not been included in this paper.

TABLE II. RESPIRATORY RATE OF RAT HEART SLICES AT VARIOUS OXYGEN CONCENTRATIONS

O_2:	100%	80%	60%	50%	40%	30%	21%
Number of experiments	279	35	35	24	20	31	233
Q_{O_2} (averages)	17.4	18.2	16.9	14.7	12.2	11.6	9.9
Standard deviation	±3.0	±2.7	±2.6	±1.8	±1.9	±1.9	±1.9
P: (as compared to Q_{O_2} in 100 per cent O_2)		>0.05	>0.05	<0.01	<0.01	<0.01	<0.01

In order to compare the effect of reduced oxygen concentration on heart muscle slices to that on other tissues, the respiration of rat kidney, liver, and spleen slices and of diaphragm was examined in 100 per cent O_2 and in 21 per cent O_2. The thickness of the slices and of the diaphragm was corresponding to that of the heart slices (average: 0.41, range: 0.31 to 0.50 mm.). Table III shows that the effect of the same reduction in oxygen concentration on tissue slices of the same thickness is considerably greater in heart muscle, kidney, and diaphragm than in liver and spleen.

TABLE III. EFFECT OF REDUCED OXYGEN CONCENTRATION ON THE RESPIRATORY RATE OF VARIOUS RAT TISSUES

	Q_{O_2} AVERAGE AND S.D.*		DECREASE	
	100 PER CENT O_2	21 PER CENT O_2	PER CENT	P
Heart	17.4 ± 3.0 (271)	9.7 ± 1.8 (217)	44	<0.01
Kidney	18.0 ± 1.4 (43)	9.3 ± 1.7 (32)	48	<0.01
Liver	7.9 ± 1.0 (17)	6.2 ± 0.7 (23)	22	<0.01
Spleen	11.1 ± 1.1 (18)	8.5 ± 1.7 (21)	23	<0.01
Diaphragm	9.1 ± 1.5 (30)	4.8 ± 0.8 (17)	47	<0.01

*The number in parantheses indicates the number of experiments.

Is Diffusion the Limiting Factor?—There is a decreased oxygen uptake with lowered oxygen concentration, as shown in Table III for heart, kidney, liver, spleen, and diaphragm. Heart, kidney, and diaphragm exhibit a greater decrease than liver and spleen. These results suggest that diffusion may not be the only limiting factor, and that a direct dependence of these tissues on oxygen concentration may exist.

This question was examined in two ways. If the lower Q_{O_2} under conditions of reduced oxygen concentration is due to inadequate diffusion, then part of the cells are under anaerobic conditions. Anaerobic glycolysis (lactic acid production) can be determined by measuring lactate concentration in the

suspension fluid. The determinations showed that, with decreasing oxygen concentration and decreasing Q_{O_2}, the lactate concentration increased (Table IV).

TABLE IV. LACTIC ACID PRODUCTION BY HEART SLICES IN 100 PER CENT OXYGEN AND IN AIR (Calculated for Successive Periods of Incubation and Expressed as 60-Minute Production)

PERIODS OF INCUBATION (MINUTES)	γ LACTIC ACID PRODUCED BY 1 MG. (DRY) TISSUE	
	100 PER CENT O_2 (AVERAGES OF 10 EXPERIMENTS)	21 PER CENT O_2 (AVERAGES OF 10 EXPERIMENTS)
0-7	76	76
7-12	6	44
12-17	0	34
17-27	0	16
27-37	0	25
37-47	0	36
47-70	0	17

The results were grouped according to the thickness of the slices (Table V). Experiments with slices thicker than 0.5 mm. were added which were not included in the calculations of Q_{O_2} averages listed above. The maximal slice thickness, which is compatible with optimal respiration at a given oxygen concentration, can be calculated according to Warburg's formula.[9] The use of this formula would define the limiting thickness in our experiments as follows: 0.43 mm. for 100 per cent O_2; 0.37 mm. for 80 per cent O_2; 0.33 mm. for 60 per cent O_2; 0.33 mm. for 50 per cent O_2; 0.32 mm. for 40 per cent O_2; 0.29 mm. for 30 per cent O_2; and 0.26 mm. for 21 per cent O_2. This theoretical consideration might not be fully applicable to heart slices[10] because of the particular anatomic structure of this tissue.

TABLE V. INFLUENCE OF SLICE THICKNESS ON OXYGEN CONSUMPTION OF RAT HEART MUSCLE AT VARIOUS OXYGEN CONCENTRATIONS

THICKNESS OF SLICES (MM.)	OXYGEN (PER CENT)						
	100	80	60	50	40	30	21
0.18-0.30	17.2	18.7	19.1		15.0	11.0	11.5
0.31-0.40	17.5	16.6	17.5	15.3	11.3	11.2	10.4
0.41-0.50	17.3	18.8	16.6	14.6	12.3	11.9	9.3
0.51-0.60	16.3	17.7	15.6	14.3	13.6	11.4	8.8
0.61-0.70	15.9	15.3	14.9	14.5	10.9	10.3	8.5
0.71-0.80	15.7	17.1	15.3	13.1			6.9
0.81-0.90	14.2						
0.91-1.00	10.2			12.7		9.0	
1.01-1.24	9.1						3.8

A comparison of the vertical columns of Table V with the calculated limiting thicknesses as listed in the preceding paragraph shows that there is a correlation between the limiting thickness for each oxygen concentration and the point where the Q_{O_2} starts to decrease, except for some groups in low oxygen concentrations.

It is concluded from these observations that insufficient oxygen diffusion is responsible for the reduced oxygen consumption which is found in heart slices under the influence of reduced oxygen concentrations.

DISCUSSION

Tissues in vitro are under conditions different from those in vivo, no matter how closely the in vivo situation is simulated. The resting state of a slice in vitro is particularly abnormal for the heart muscle which, in vivo, never rests. The metabolism of the irritable heart muscle tissue might be altered by the preparation of slices. It has been stated that heart slices are not suitable for metabolic studies.[11] The wide variation of Q_{O_2} found in the literature seemed to support this theory. Our investigations showed that the slice technique is applicable for the measurement of heart muscle metabolism provided the experimental conditions are optimally standardized.

The average Q_{O_2} of 17.4, which we found with optimal oxygen supply and diffusion and with an optimal condition of the slice, is higher than most values reported in the literature although occasional figures of 17,[12] 18,[13] or 21[7] occur. The explanation lies most probably in the condition of the slices and in the composition of the suspension fluid. We used a single slice with a weight, after drying, of 5 to 7 mg. whereas, for example, in the investigations resulting in a Q_{O_2} average of 12.5,[5] 3 or 4 slices with a total dry weight of 3 to 5 mg. were used. It is obvious that in these experiments the ratio of damaged surface cells to undamaged cells in the inner part of the slices is high. We obtained similar results when slices of similar condition were used.

Cooling of the tissue before and during the slicing procedure has been recommended by a number of authors as being essential.[14] We did not apply it because, in preliminary experiments, no difference was apparent in the initial respiratory rate whether whole heart and slices were not cooled at all or were cooled in the icebox for varying periods up to 30 minutes. After more than 30 minutes' cooling, the respiration was depressed from the beginning, and even after 5 minutes' cooling, a progressive decline of the respiratory rate was found.

It has been shown before that the metabolism of heart tissue in vitro is very sensitive to anoxia,[6, 15] and it has also been mentioned that its respiration is more inhibited than the respiration of other organs.[16, 17] Our results are in agreement with these observations.

A direct effect of oxygen concentration upon cell respiration would be in contrast to the all-or-nothing axiom of Warburg[18] and Meyerhof.[19] They assumed that the rate of respiration of cells is maximal as long as any oxygen is available. It has been shown by Kempner[20-22] that this is not the case. Thus, in the course of the present experiments, it was not considered impossible that the oxygen consumption of cardiac muscle might be directly dependent on oxygen concentration per se. However, it was seen that the apparent dependence was caused by insufficient oxygen diffusion into the tissue.

It might be of interest to compare the Q_{O_2} values obtained from slices of hearts in vitro with the oxygen consumption of the whole heart. Various methods have been applied to estimate the oxygen consumption of the whole heart or the left ventricle in experimental animals and man. Some Q_{O_2} values, calculated in the same manner as in our experiments, are: 14 for heart-lung

preparation of dogs[23]; 21 for whole heart of morphinized dogs[24]; 23 for that of anesthetized dogs[25]; varying between 23 and 58 for that of unanesthetized dogs[26]; 19 for left ventricle of man[27]; and 18 and 21 for whole heart of man.[28, 29] These figures refer to the beating heart; the value for the resting tissue must be expected to be lower.

SUMMARY

1. The question whether the manometric methods of Warburg can be used for studies of heart muscle metabolism has been re-evaluated. It is concluded that the slice technique is applicable provided the experimental details are optimally standardized.

2. The basal respiratory rate (Q_{O_2}) of normal rat heart muscle under optimal conditions regarding oxygen supply, diffusion, and state of the slices was found to be higher than most values reported in the literature. In 279 experiments, it averaged 17.4, with a standard deviation of ± 3.0.

3. With lower oxygen concentrations, the Q_{O_2} decreases. In 80, 60, 50, 40, 30 and 21 per cent oxygen, the average Q_{O_2} values were 18.2, 16.9, 14.7, 12.2, 11.6, and 9.9, respectively.

4. Heart slices, as well as kidney slices and diaphragm, showed a greater dependence on oxygen concentration than liver and spleen slices.

5. The question whether the apparent dependence of the respiratory rate on oxygen concentration is due to the effect of the oxygen concentration per se or to gas diffusion, has been studied. It is concluded that insufficient oxygen diffusion in the tissue is responsible.

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EFFECT OF DIGITALIS GLYCOSIDES ON THE OXYGEN CONSUMPTION OF HEART MUSCLE IN VITRO

ERNST PESCHEL, M.D., AND CLOTILDE SCHLAYER, PH.D.
DURHAM, N. C.

THE specific action on the heart of the glycosides of the digitalis group is clear from the clinical point of view, but the mechanism of this action is still obscure. Changes in cellular metabolism must occur, of course, but little is known in regard to this.

Increase in oxygen consumption is a change that might logically be expected. But it is not definitely established whether cardioactive glycosides increase the oxygen consumption, or the utilization of substrates, by the heart muscle cells. It is widely assumed that they do not. In recent studies on the whole heart in vivo, applying coronary sinus catheterization techniques, no significant difference in oxygen consumption or pattern of substrate utilization was found between the normal and the failing heart[1, 2] and between the digitalized and the nondigitalized heart.[3] It was concluded that apparently the production of energy is not different but that the available energy might be differently utilized because of changes in the contractile proteins of the heart muscle.[2] The problem is still one of cellular metabolism, and it appears that further studies using the manometric methods of Warburg should be made.

The results obtained by manometric measurements of the effect of digitalis glycosides on myocardial metabolism had been summarized up to 1949, and they showed that there was undoubtedly an increase in oxygen consumption under the influence of these agents.[4] Stimulation of respiration, followed by depression, was again described in 1950.[5] On the other hand, it was reported in 1951[6] that no significant increase could be found, and, in 1953,[7] that an increase was demonstrable but only after depression of the original respiratory rate by preincubation or by treatment with barbiturate. In 1956, it was stated once more that the reports on oxygen consumption measured with Warburg's methods are conflicting.[2]

In view of these discrepancies, and based upon experiences with heart slices reported in a preceding paper,[8] the problem was re-examined to see whether an increase of oxygen consumption is associated with the action of

From the Department of Medicine, Duke University Medical Center, Durham, N. C.

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digitalis glycosides on heart muscle in vitro, and whether a difference could be demonstrated for the action of these substances on heart muscle as compared to other muscle.

METHODS AND MATERIAL

Details of the methods applied are the same as described in the previous paper.[8] Rats of the Osborne-Mendel strain were used. The digitalis preparations were strophanthin G (Ouabaine, Arnaud) and desacetyllanatoside C (Cedilanid, Sandoz), commercially supplied in ampules containing 0.25 mg. in 1 ml. and 0.8 mg. in 4 ml., respectively. Suspension fluid was the modification of Ringer's solution described by Levy,[9] with 0.01 M glucose. Care was taken that the concentration of the suspension fluid was adjusted in case of admixtures so that the final ionic composition remained unaltered. All experiments were done with the direct method of Warburg,[10] using 100 per cent oxygen as the gas phase. Readings were taken at 10-minute intervals, usually over a two-hour period. Because of the fluctuations in the basal respiration of heart muscle in vitro which had been observed from heart to heart but much less from slice to slice of the same heart,[8] all studies of the digitalis effect were done with simultaneous controls using slices from the same heart. Each individual Q_{O_2} value was derived from duplicates or triplicates.

RESULTS

A representative experiment concerned with the action of ouabain on the oxygen consumption of heart slices is described in Table I. Q_{O_2} indicates $mm.^3$ oxygen consumed in one hour by 1 mg. dry weight of tissue.

TABLE I. ACTION OF OUABAIN ON OXYGEN CONSUMPTION OF HEART SLICES: PROTOCOL OF AN INDIVIDUAL EXPERIMENT

		NO OUABAIN		OUABAIN (50 γ/ML.)	
Slice		1	2	3	4
Dry weight (mg.)		5.3	4.5	5.5	4.4
Thickness (mm.)		0.50	0.42	0.48	0.50
Q_{O_2} (calculated from 30-minute periods)	1	17.1	17.2	20.8	21.0
	2	16.5	16.9	20.1	19.9
	3	16.8	17.0	18.7	21.8
	4	17.1	17.0	18.4	19.7
Average (period 1)		17.2		20.9	
Per cent change by ouabain				+21.5 per cent	

The effects of various dosages of ouabain are listed in Table II. An ouabain concentration of 2.5 γ/ml. (3.3×10^{-6} M) was without any effect; a concentration of 5 γ/ml. (6.6×10^{-6} M) caused a slight stimulation. In the range from 10 to 50 γ/ml. (1.3 to 6.6×10^{-5} M), a significant stimulation of the oxygen consumption was found, increasing with dosage. It decreased or disappeared frequently after the first hour of incubation. This stimulation varied insofar as, at each dosage level, there were a number of instances where the tissue did not respond to the glycoside. These experiments are included in the calculation of averages listed in Table II. A dose of 100 γ/ml. (1.3×10^{-4} M) did not stimulate; it had a depressing effect in half of the cases.

For comparison, the effect of ouabain had been studied on the oxygen consumption of diaphragm and of kidney and liver slices. The dose maximally increasing the Q_{O_2} of heart muscle (50 γ/ml.) had a slightly depressing effect on kidney and liver respiration (Table III) which was statistically significant in the case of liver.

The effect of ouabain on diaphragm was studied more extensively in order to compare striated skeletal muscle with heart muscle. There was a depressing effect on the oxygen consumption of diaphragm, increasing with the dosage of ouabain (Table IV). This depression was persistent and, in higher dosages, progressive.

TABLE II. ACTION OF OUABAIN ON OXYGEN CONSUMPTION OF HEART SLICES: EFFECT OF VARIOUS OUABAIN CONCENTRATIONS

DOSAGE OF OUABAIN (γ/ML.)	NUMBER OF EXPERIMENTS	Q_{O_2} AVERAGES AND S.D. WITHOUT OUABAIN	Q_{O_2} AVERAGES AND S.D. WITH OUABAIN	CHANGE (PER CENT)	SIGNIFICANCE OF CHANGE (P)
2.5	9	17.5 ±2.6	17.4 ±2.2	0	
5	7	17.0 ±2.2	17.7 ±1.7	+ 4.1	> 0.5
10	41	17.5 ±2.1	19.6 ±3.1	+12.0	< 0.01
25	39	16.7 ±2.3	19.1 ±2.7	+14.4	< 0.01
50	57	17.7 ±2.0	21.0 ±3.2	+18.6	< 0.01
100	46	17.3 ±2.8	16.7 ±4.5	− 3.5	$0.5 > p > 0.4$

TABLE III. EFFECT OF OUABAIN ON OXYGEN CONSUMPTION OF KIDNEY AND LIVER SLICES

	NUMBER OF EXPERIMENTS	Q_{O_2} AVERAGES AND S.D. WITHOUT OUABAIN	Q_{O_2} AVERAGES AND S.D. WITH OUABAIN	CHANGE (PER CENT)	SIGNIFICANCE OF CHANGE (P)
Kidney	9	18.6 ±0.9	18.0 ±1.1	− 3	$0.3 > p > 0.2$
Liver	8	9.3 ±1.0	8.0 ±0.8	−14	$0.05 > p > 0.02$

TABLE IV. EFFECT OF OUABAIN ON OXYGEN CONSUMPTION OF DIAPHRAGM IN VITRO

DOSAGE OF OUABAIN (γ/ML.)	NUMBER OF EXPERIMENTS	Q_{O_2} AVERAGES AND S.D. WITHOUT OUABAIN	Q_{O_2} AVERAGES AND S.D. WITH OUABAIN	CHANGE (PER CENT)	SIGNIFICANCE OF CHANGE (P)
0.01	5	11.6 ±1.5	11.6 ±1.5	0	
0.02	10	10.2 ±1.4	9.8 ±1.4	− 4	> 0.5
0.1	7	12.0 ±1.1	10.5 ±1.0	−13	$0.02 > p > 0.01$
0.5	15	12.4 ±1.2	9.8 ±0.8	−21	< 0.01
2.0	16	11.1 ±1.2	8.7 ±0.8	−22	< 0.01
5.0	4	11.2 ±0.5	8.8 ±0.6	−21	< 0.01
10.0	5	11.5 ±1.4	8.3 ±1.0	−28	< 0.01
25.0	3	11.0 ±1.0	7.8 ±0.5	−29	< 0.01
50.0	4	12.2 ±0.7	8.0 ±0.5	−34	< 0.01

As a representative of cardiac glycosides other than ouabain, Cedilanid was used on heart slices and diaphragm. It had, in comparable concentrations, very similar effects on diaphragm; on heart, the effect was similar in principle but different in regard to concentrations (Table V).

TABLE V. COMPARISON OF EFFECTS OF OUABAIN AND CEDILANID ON OXYGEN CONSUMPTION OF HEART AND DIAPHRAGM IN VITRO

GLYCOSIDE CONCENTRATION (γ PER ML. OF SUSPENSION FLUID)		CHANGE IN OXYGEN CONSUMPTION (PER CENT)			
		HEART SLICES		DIAPHRAGM	
OUABAIN	CEDILANID	OUABAIN	CEDILANID	OUABAIN	CEDILANID
2.5		0		−22	
	4		0		−21
	8		+ 8		
5		+ 4		−21	
	10		+11		
	16		+18		
10		+12		−28	
	20		+ 5		
25		+14		−29	
	40		− 1		
50		+19		−34	
	80		−50		−38
100		− 4			

DISCUSSION

Oxygen consumption of rat heart slices was stimulated by digitalis glycosides in the dosage range of 10^{-5} M. This is in agreement with those former reports in which an effect had been described.[4, 11, 12] The doses are high compared with the dose known to be effective in human patients; on a tissue-weight basis (γ per 100 mg. wet tissue), it is 3,000 times as much. However, the tolerance of animals to drugs cannot be compared quantitatively with those of human patients. The typical action of atropine in the rat, for instance, is produced by 100,000 times the dosage active and tolerated in man.[13] In regard to digitalis glycosides, the rat is known to have a high resistance; doses like those applied here in vitro have been tolerated well by living rats[4, 11]; the LD_{50} dose is 5 to 10 times higher than the dose used in the present experiments.[13]

Such a difference in the dosage required to elicit a certain response does not invalidate either the observations, or attempts to transfer them from animal to human tissue. However, the question has been frequently raised whether results obtained in vitro can be applied to the situation in vivo, in particular whether the action of cardiac glycosides on heart slice respiration in vitro is of relevance with regard to the action on the beating heart in vivo. This question has been answered affirmatively by Wollenberger,[4] on the basis of literature data and his own observations.

A glycoside concentration of the order of 10^{-4} M seems to represent the border line between stimulating and toxic action on rat heart slices. A stimulation was observed by one group of investigators,[9] inhibition by another,[14] and, in the present set of experiments, stimulation was found in some and depression in other experiments.

The selective affinity of digitalis to heart muscle and the possibility of demonstrating this in vitro is strongly emphasized by the present experiments. Not only is there a stimulation of the oxygen consumption of heart slices which was not seen in the other tissues tested, but the oxygen consumption of other tissues is depressed by digitalis glycosides. This is most pronounced and of particular interest in the case of diaphragm where a statistically significant depression was found down to a glycoside concentration $1/500$ of that which stimulates the oxygen consumption of heart slices.

The depression by digitalis glycosides of the oxygen consumption of tissues other than heart, particularly of skeletal muscle, is an indication of temporary cell damage. This might explain the observation that digitalis in man in therapeutic dosages mobilizes intracellular potassium without effect on the sodium and potassium balance of the heart—a finding that had been interpreted as suggesting potassium liberation primarily from extracardiac tissues.[15]

The condition of the slices which are used for experiments like those discussed here is of decisive importance. Homogenized or minced heart tissue does not show an effect of digitalis glycosides[4]; the structure of cells must be intact to evoke any change in oxygen consumption. One of the characteristics of unsatisfactory slices is that an undue number of cells is structurally damaged. The difficulties in obtaining satisfactory slices from heart tissue probably explain the conflicting results reported on manometric measurements of myocardial metabolism, including the effect of digitalis glycosides on oxygen consumption.

SUMMARY

Digitalis glycosides stimulate the oxygen consumption of rat heart slices by 12 to 19 per cent. The effective concentration is in the order of 10^{-5} M.

The oxygen consumption of other tissues, such as kidney and liver, is not stimulated but slightly depressed by corresponding concentrations of the glycoside.

The oxygen consumption of diaphragm, an example of striated skeletal muscle, is depressed by even very small concentrations of digitalis glycosides. The depression, increasing with dosage, is considered an indication of temporary cell damage. This might explain the liberation, by therapeutic digitalis doses in man, of intracellular potassium without effect on sodium and potassium balance of the heart.

It is suggested that the conflicting results reported on manometric measurements of the effect of digitalis glycosides on heart are due to the difficulties in obtaining satisfactory slices from heart tissue.

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DIURETIC EFFECTS OF CHLOROTHIAZIDE IN TOXEMIA OF PREGNANCY

N. S. Assali, M.D., L. Judd, B.S., N. Mondz, B.A., and K. Dasgupta, M.D.
Los Angeles, Calif.

With the Technical Assistance of M. Ross and L. Friedman

ANIMAL experiments[1, 2] have shown that chlorothiazide (6-chloro-7-sulfamyl-1,2,4-benzothiadiazine-1,1-dioxide) inhibits renal reabsorption of sodium and chloride and increases their excretion together with water. In vitro, this compound acts as a carbonic anhydrase inhibitor, but in vivo, unless large doses are employed, such an action is not clearly evident since bicarbonate in blood and urine is slightly altered while chloride is the main anion excreted.[1] These properties prompted investigators to explore the usefulness of this drug in the management of edematous states. The reports have indicated that oral administration of chlorothiazide in doses varying between 250 to 2,000 mg. daily is followed by weight loss and increased excretion of water, sodium, chloride, and, to a certain extent potassium.[3-8] Some authors have suggested that the drug is also of some benefit in the treatment of hypertension either because of the synergistic effect of other vasodepressor drugs or by virtue of its effects on sodium metabolism and of a true hypotensive action.[9, 10]

The present reports deals with studies of the diuretic and antihypertensive effects of the oral and intravenous administration of chlorothiazide to patients with toxemia of pregnancy.

MATERIAL AND METHODS

The studies were carried out on 15 pregnant subjects with toxemia of pregnancy. None of these patients had history of any previous cardiovascular renal disease. The diagnosis of this disease was based on the appearance after the twentieth week of gestation of a persistent hypertension (over 140/90 mm. Hg), edema varying from 1 to 4 plus, and, in certain instances, proteinuria. No effort was made to classify these cases as having mild or severe toxemia since the criteria for such a classification have proved to be unsatisfactory. The ages of the patients varied from 20 to 36 years, and the duration of pregnancy varied from 24 to 38 weeks. All the patients were at bed rest in the hospital without any specific medication throughout the periods of study.

The effects of oral chlorothiazide were studied on 11 patients; 10 were on a daily diet containing 85 mEq. and one on a diet containing 8.5 mEq. of sodium as sodium chloride. The studies comprised 3 consecutive periods. The first period lasted from 3 to 5 days and served as a control. During the second period, which lasted from 4 to 6 days and

From the Department of Obstetrics and Gynecology, University of California Medical Center, Los Angeles, Calif.

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followed immediately after the first, chlorothiazide tablets were administered in daily doses varying from 500 mg. to 2,000 mg. (8 to 33 mg./Kg. of body weight) divided into 2 doses per day. The third period served as a postcontrol (the recovery period) and varied from 3 to 4 days. Two patients had additional periods of 3 days of chlorothiazide treatment with double the dose used in the first period and 3 days of recovery, starting

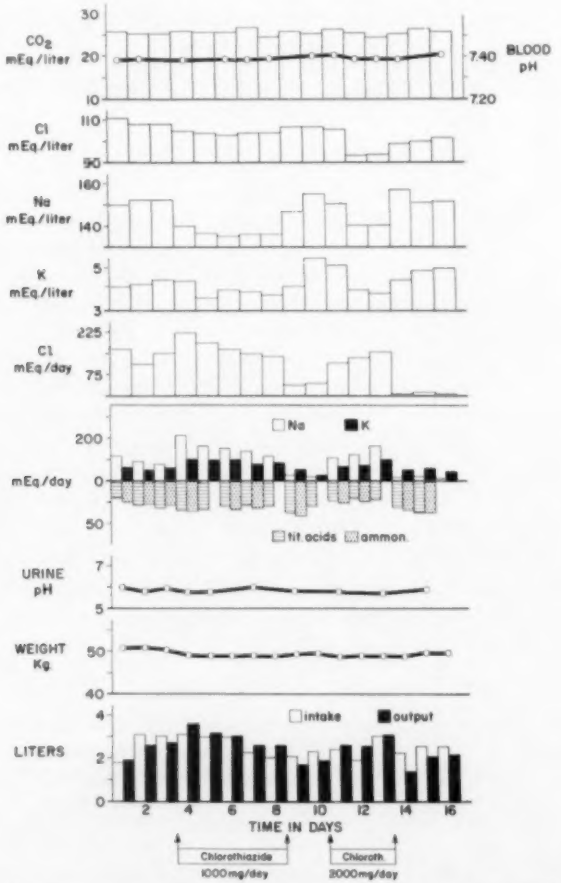

Fig. 1.—Data on blood and urine composition of one patient with toxemia of pregnancy who received two courses of chlorothiazide alternated with recovery periods. During the first course, electrolyte excretion increased while plasma electrolytes fell. A typical electrolyte retention with a rise in plasma electrolytes occurred during the first recovery period. A second course of treatment with double the first dose reinitiated the diuretic effects and produced a marked fall in plasma electrolytes. During the second recovery period, sodium and chloride nearly disappeared from the urine while potassium continued to be excreted at a near control rate.

on the second day of the first recovery period (Fig. 1). This experiment was designed to determine whether this drug would overcome the electrolyte retention which occurs upon cessation of therapy, and whether doubling of the dose would result in a greater increment of electrolyte excretion.

During all these periods, the patient's weight and her fluid intake and output were recorded daily. Blood pressure (sphygmomanometer) and pulse rate were recorded 4 times a day by the same observer while the patient was recumbent in bed. Daily arterialized blood samples were drawn under oil without stasis from a superficial antecubital vein after soaking the arm in warm water for 10 to 15 minutes. Blood collected in this way is closer to arterial blood and, therefore, gives better pH and CO_2 values than true venous blood. Twenty-four hour urine specimens preserved with toluene were collected throughout the study and served for analysis of electrolytes. For the determination of urinary pH, ammonia, and titratable acids, a catheterized specimen was obtained under oil at the same time each day. The completeness of the urine collections in the 24-hour samples was checked by the creatinine excretion.

The effects of intravenous administration were investigated in 4 patients. The studies were performed in the postabsorptive stage with the patient lying in the recumbent position. A priming dose of inulin and para-aminohippurate (PAH), calculated for each patient according to her body weight, was injected intravenously and was followed by a constant infusion aimed at maintaining plasma levels of 200 mg. per liter of inulin and 30 mg. per liter of PAH. An equilibration period of 30 minutes was allowed, and then 3 to 4 thirty-minute control clearance collections were obtained. Thereafter, 5 or 10 mg./Kg. of body weight of chlorothiazide (2 patients received 5 and 2 received 10) were injected intravenously in a single dose over a period of 1 to 2 minutes and 3 to 4 thirty-minute clearance collections were obtained. Two blood samples were withdrawn at the mid-point of each clearance period (one anaerobically for determination of pH and CO_2). Urine specimens were collected under oil for determination of pH and CO_2.

Blood and urine pH, CO_2 content (CO_2 of the 24-hour urine specimens was not analyzed), HCO_3^-, Na, Cl, K, inulin and PAH, and urinary ammonia and titratable acids were determined by methods previously described.[11] Osmolality of plasma and urine were determined by freezing point depression and osmolal and free water clearances were calculated by the method of Smith.[12]

RESULTS

General Effects.—In the doses given, chlorothiazide either orally or intravenously was well tolerated by all the patients. Gastrointestinal disturbances were not observed even at oral doses as high as 2,000 mg. per day.

The effects of oral chlorothiazide on blood and urine composition: Table I presents data obtained from a patient with toxemia of pregnancy who received 1,000 mg. of chlorothiazide orally (500 mg. in the morning and 500 mg. in the afternoon) and a diet containing 85 mEq. of Na per day. The data on this patient will be treated as a typical example of a group of 6 out of 10 patients who responded in a similar manner. These 6 patients had pitting edema varying from 2 to 4 plus.

In this group of patients, the drug had no significant effects on blood pH although blood CO_2 content fell slightly. Plasma Na and Cl did not change significantly whereas plasma K fell 1 to 1.4 mEq. per liter (Table I).

On the first day of treatment, the excretion of Na and Cl more than tripled. Although on the second, third, and fourth day it fell somewhat, it was still higher than control values (Table I). During the last day of the treatment period, the excretion of these ions had nearly returned to pretreatment values. Upon cessation of therapy, a marked retention of Na and Cl occurred but the total amount retained during the 3 days of the recovery

TABLE I. CHANGES IN WEIGHT, URINE OUTPUT, BLOOD PRESSURE, AND URINE AND BLOOD COMPOSITION INDUCED BY CHLOROTHIAZIDE (PATIENT M. F.)

						BLOOD					URINE		
PERIOD	DAY	WEIGHT (KG.)	INTAKE (C.C.)	OUTPUT (C.C.)	B.P. (MM. Hg)	pH	CO_2 mEq./L.	Na mEq./L.	K mEq./L.	Cl mEq./L.	Na mEq./DAY	K mEq./DAY	Cl mEq./DAY
Control	1	97.4	1,898	1,725	160/100	7.41	22.4	136	3.3	110	99.2	22.4	99.2
	2	97.2	2,303	1,680	150/94	7.43	22.7	136	4.0	108	98.5	26.5	91.2
	3	97.2	3,409	3,010	140/90	7.40	21.2	140	3.9	105	106.9	37.9	102.0
Treatment	5	97.0	2,284	2,980	140/90	7.43	21.0	140	3.5	102	337.0	57.2	291.0
	6	96.2	2,619	2,630	132/86	7.41	21.4	140	2.9	105	235.0	81.0	234.6
	7	95.3	2,474	2,735	140/90	–	–	–	–	–	185.6	99.3	196.2
	8	94.6	3,519	3,509	142/92	7.40	20.9	138	2.5	107	118.8	85.0	140.3
	9	94.6	3,200	3,109	136/88	7.42	21.0	137	3.0	104	116.0	65.0	112.0
Recovery	10	95.0	3,004	1,720	140/90	7.42	22.5	137	3.2	102	10.3	33.5	12.2
	11	96.2	3,194	2,560	132/84	7.43	–	136	3.1	102	24.8	30.2	19.7
	12	96.5	3,794	2,600	134/84	7.41	23.6	136	3.2	103	35.4	30.4	23.3

period was far below the increment excreted during chlorothiazide administration (Table I). The excretion of K also increased significantly throughout the period of treatment and returned to control values upon cessation of therapy (Table I). Urine pH and the excretion of titratable acids and ammonia (not listed in Table I) remained practically unchanged.

Concomitant with the increase in electrolyte excretion, there was an increase in urine output and loss of weight (Table I). However, the increment in urine flow was proportionally less than that of electrolyte excretion.

Fig. 1 illustrates data obtained from one patient who received 2 courses of chlorothiazide alternated with recovery periods. The data obtained during the first course of therapy will be taken as a typical example of the second group of 4 patients who responded in a similar manner. The edema in these 4 patients was somewhat less than in the other 6 patients discussed above.

The most striking difference between the response of these 4 patients and that of the other 6 represented by the patient in Table I was the change in plasma electrolytes. As depicted in Fig. 1, plasma Na, K, and Cl fell significantly during the first course of chlorothiazide therapy, despite the fact that the dose employed was the same. The increment in the excretion of these ions observed during the first day of therapy was not significantly different from that of the other group, but the fall in their excretion on subsequent days was somewhat sharper. The changes in blood pH and CO_2 content, as well as in urine pH and in the excretion of ammonia and titratable acids were similar to those of the other group.

During the 2 days of the recovery period, a typical retention of electrolytes occurred with a concomitant rise in plasma Na, K, and Cl. The administration of another course of chlorothiazide in double the first dose resulted in another rise in electrolyte excretion which was of less magnitude than that observed during the first course. Plasma electrolytes fell considerably during the second course of treatment. Upon cessation of therapy, Na and Cl nearly disappeared from the urine while their concentration in the plasma rose; the excretion and plasma concentration of K returned to pretreatment levels.

In this group of cases, there was also a rise in urine output and loss of weight, but here again the increment in urine flow was less striking than that of electrolyte excretion.

Figs. 2, 3, and 4 present the mean excretion of Na, Cl, and K for the 10 patients who received 85 mEq. of sodium in the diet. The vertical bars represent one standard deviation of the mean. In addition, Figs. 2 and 3 present the mean concentration of Na and Cl in the urine of these patients. In these figures, the values for the last day of the control period were taken as zero base line and the other values during the control, treatment, and recovery periods were plotted as changes (Δ values) from the last control day. In this way it was possible to average the data obtained from the various patients treated in the same manner.

It is clear from these figures that the peak of electrolyte excretion occurred during the first day of the treatment period and that the response to

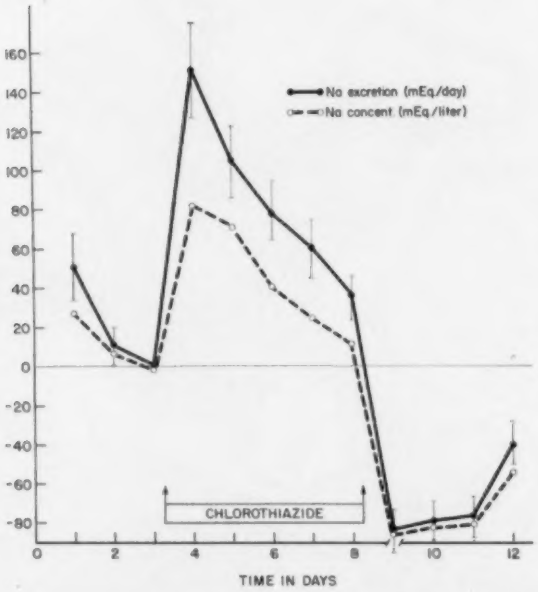

Fig. 2.—Mean excretion and concentration of sodium of the 10 patients who received 85 mEq. of sodium per day. The vertical bars represent one standard deviation of the mean. The last control day was taken as zero base line and the values for the other days were plotted as changes (Δ values) from this base line. Note the marked increase in the excretion and concentration of sodium which occurred on the first day of therapy. Both excretion and concentration declined progressively on subsequent days. During the recovery period, a striking sodium retention occurred but the quantity retained was far below the increment excreted during therapy.

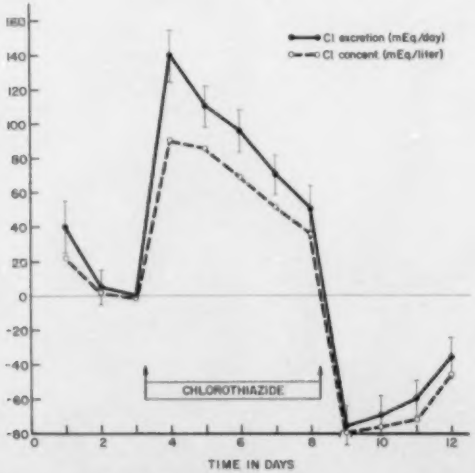

Fig. 3.—Mean excretion and concentration of chloride of the same patients represented in Fig. 2. Note the parallelism between the behavior of chloride and sodium.

the drug decreased progressively until the end of the treatment period. It is also evident that the major component in the changes in electrolyte excretion during the treatment and recovery periods was the change in their concentration in the urine and not in the urine flow. The data also show that although potassium excretion increased in all patients treated, chloride and sodium were the chief ions eliminated.

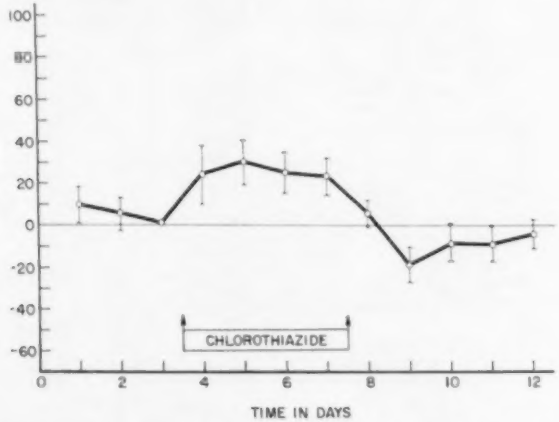

Fig. 4.—Mean excretion of potassium (mEq. per day) of the 10 patients represented in Figs. 2 and 3. Although potassium excretion increased in nearly all cases, the increment was less than that of sodium and chloride. Similarly, the retention of potassium in the recovery period was less marked than that of sodium and chloride.

Fig. 5 presents data from the patient who received a daily dose of 1,000 mg. of chlorothiazide and a diet containing 8.5 mEq. of Na per day. A striking fall in plasma electrolytes occurred promptly after the initiation of therapy and remained so throughout the entire treatment and recovery periods. During these same periods, plasma CO_2 content and, to a certain extent, blood pH rose above control values.

The excretion of Na and Cl increased significantly during the first day of treatment. Although on the second day the excretion of these ions had returned to pretreatment values, on the third, fourth, and fifth day of treatment it was definitely below control values. During the recovery period, these ions could hardly be detected in the urine. The excretion of K increased significantly during the first 2 days of therapy and remained above control values for the duration of the experiment. A continuous loss of weight occurred during the entire treatment and recovery periods.

The effects of oral chlorothiazide on blood pressure and pulse rate: In the doses given and for the duration of the experiments, the drug did not have a significant vasodepressor action. A slight fall in blood pressure occurred in 4 patients but this fall could not be correlated with the periods of drug administration and, therefore, might have been due to bed rest alone. Similarly, the drug had no action on the pulse rate.

Dose-response relationship: Orally and in the range of dose utilized in these studies (500 to 2,000 mg. daily) it was difficult to establish a dose-response relationship. The effects observed after 2,000 mg. were not significantly different from those of 500 mg.

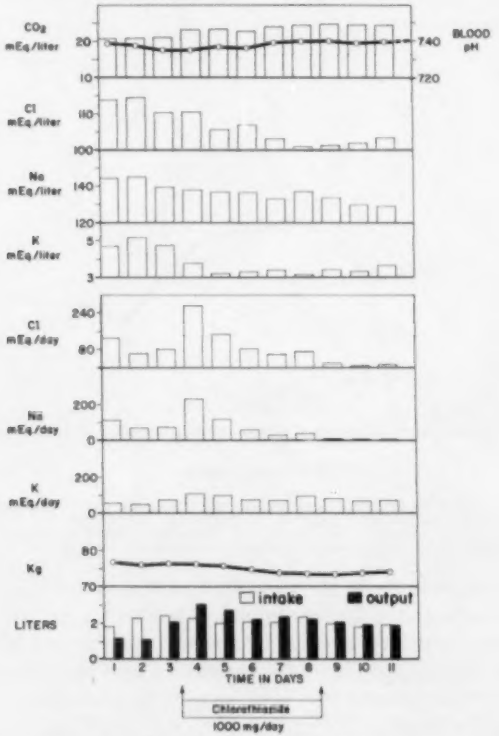

Fig. 5.—Data obtained from one patient with toxemia studied while receiving a daily diet containing 8.5 mEq. of sodium. Note the marked decrease in plasma electrolytes which occurred during treatment and persisted after cessation of therapy. A certain degree of alkalosis occurred at the same time. The excretion of sodium and chloride increased for only one day after the initiation of treatment but progressively fell to extremely low values on subsequent days. However, potassium excretion increased and remained elevated throughout the entire experiment. Replacement therapy of electrolytes was not required in this patient.

Effects of intravenous administration: Figs. 6 and 7 show data obtained from one patient who received a single intravenous dose of 10 mg. chlorothiazide per kilogram of body weight. The other patient who received the same dose responded in a similar manner.

The effects of intravenous chlorothiazide on blood pressure, renal plasma flow (PAH clearance), and glomerular filtration rate (inulin clearance) were not significant (Fig. 6).

Osmolal clearance (C_{osm}) rose significantly after the injection of the drug and remained elevated for the duration of the experiment. Free water clearance (C_{H_2O}) increased during the first collection period after the injection of the drug but became negative during the 2 subsequent periods (Fig. 6).

Intravenous administration of 10 mg./Kg. of chlorothiazide evoked a tenfold increase in the excretion of Na which became evident within the first 30 minutes after the injection. The excretion of K nearly tripled. The excretion of both ions was still elevated 90 to 120 minutes after the injection

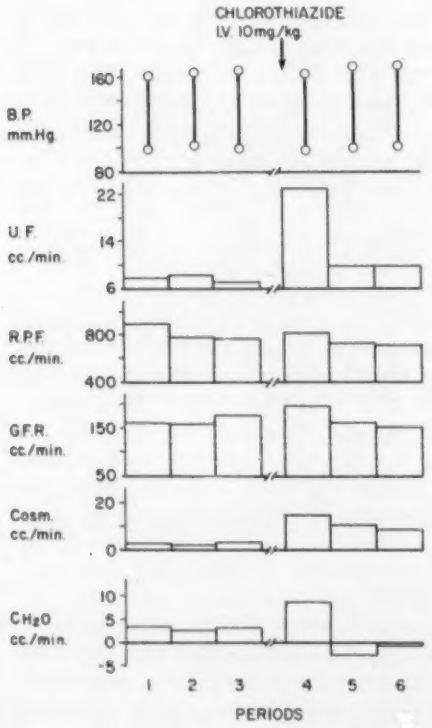

Fig. 6.

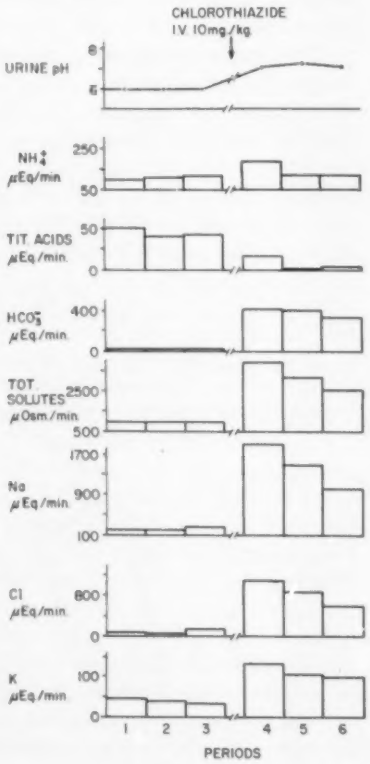

Fig. 7.

Fig. 6.—Effects of 10 mg./Kg. of chlorothiazide intravenously on blood pressure, urine flow, renal plasma flow, glomerular filtration rate, and osmolal and water clearances in a patient with toxemia of pregnancy. Collection periods before and after drug injection were of 30 minutes each. Note the negligible effects on blood pressure, GFR, and RPF. Urine flow increased markedly in the first period but fell sharply in the 2 subsequent periods. Nevertheless, osmolal clearance rose and remained elevated throughout. Free water clearance increased in the first period but became negative thereafter.

Fig. 7.—The effects of 10 mg./Kg. of intravenous chlorothiazide on urine pH, and on the excretion of ammonia, titratable acids, bicarbonate, total solutes, sodium, chloride, and potassium in the same patient as represented in Fig. 6. Note the striking increase in urine pH as well as in the excretions of HCO_3^-, total solutes, Na, Cl, and K. This increase persisted for the 3 periods after the injection despite the fact that the urine flow (Fig. 6) had fallen considerably after the first collection period.

(Fig. 7). The excretion of these cations was matched not only by an increment in Cl^- excretion as in the case of oral administration but also by HCO_3^- since the excretion of this anion increased from an average of 8 to an average of 424 μEq per minuute (Fig. 7). Total solute excretion rose markedly after the injection and remained elevated for the entire experiment despite the

fact that the urine flow increased only in the first postinjection period (Figs. 6 and 7). Urine pH rose from an average of 5.95 to an average of 7.14. The excretion of ammonia increased slightly during the first period after the drug injection but subsequently returned to control values and the excretion of titratable acids fell markedly throughout all of the postinjection periods (Fig. 7).

The injection of chlorothiazide evoked a slight fall in plasma Na and K which was more evident during the first collection period; blood pH fell 0.01 to 0.02 unit, and plasma CO_2 content fell 1 to 2 mEq. per liter. Plasma osmolality fell from an average of 284 to an average of 275 mOsm. per liter.

In the dose of 5 mg. per kilogram of body weight the drug produced a smaller increase in Na and Cl excretion, but the excretion of HCO_3^- was far below that observed after the 10 mg. dose (Table II). Urine pH increased from an average of 5.20 to 6.25.

Neither the intravenous dose of 10 mg./Kg. nor that of 5 mg./Kg. had any acute effect on blood pressure (Fig. 6).

TABLE II. COMPARISON OF THE EFFECTS OF 5 AND 10 MG./KG. OF CHLOROTHIAZIDE INTRAVENOUSLY ON THE EXCRETION OF Cl, HCO_3^-, AND Na*

COMPOUND	PERIOD	5 MG.	10 MG.
Cl	Control	40	50
	Treatment	540	1,038
HCO_3^-	Control	1	8
	Treatment	38	424
Na	Control	180	200
	Treatment	1,300	1,727

*The treatment values are those observed during the first period after the injection. Figures are in microequivalents per minute.

DISCUSSION

The present data show that chlorothiazide given orally or intravenously to patients with toxemia of pregnancy increases primarily the excretion of sodium, chloride, and potassium and secondarily the excretion of water. From the available evidence in animals and man,[1-8] it appears that such an action is caused by inhibition of reabsorption of these ions by the renal tubules. The following factors speak in favor of this hypothesis: (1) the marked rise in concentration of these ions in the urine even when the urine flow remains the same or increases slightly; (2) the absence of any significant change in glomerular filtration rate and, therefore, the filtered load of electrolytes; (3) the marked and persistent increase in total solute excretion along with the increase in osmolal clearance when the urine flow has returned to control values; and (4) the negative free water clearance. All these factors tend to indicate that the increase in urine flow is secondary to an osmotic effect evoked by an increased elimination of an osmotically active solute. The excretion of water and solute would then lead to a loss of weight.

Although the site of action of this drug is the renal tubule, the exact mechanisms of such an action are still obscure. Orally, in doses which were 3 times those used intravenously, the drug did not seem to have any action

which would suggest carbonic anhydrase inhibition. Although HCO_3^- excretion during oral administration was not determined, the lack of changes in urine pH and in the excretion of titratable acids and ammonia coupled with the absence of any significant alteration in blood pH and CO_2 content speak against any striking carbonic anhydrase inhibition. These findings are in agreement with those reported in dogs and man after oral administration.[1-7] However, when the drug is injected intravenously, there appears to be definite signs of carbonic anhydrase inhibition such as a certain degree of acidosis and a marked HCO_3^- excretion. A possible explanation of the difference in action of the oral and intravenous route is that the latter rapidly delivers to the renal tubules a high concentration of the drug which is sufficient to produce a certain carbonic anhydrase inhibition whereas the oral route does not. This possibility receives further support from the fact that, whereas the drug did not show increasing response with progressively increasing doses orally, doubling the dose intravenously resulted in a much higher increment in HCO_3^- excretion. This would suggest that the ability of this drug to inhibit carbonic anhydrase depends on the immediate blood level reaching the renal tubules. These findings are in agreement with those of others.[8] However, it should be realized that the action of this drug is more complex since despite its apparent carbonic anhydrase inhibition it still increases the excretion of chloride, a property not possessed by other true carbonic anhydrase inhibitors.

The diuretic action of chlorothiazide, when given orally, is prompt and reaches its maximum on the first day of treatment. When injected intravenously, maximum effect is observed within the first 30 minutes. In either instance, the effects of the drug become progressively less, even though in a number of cases some diuretic action was still present after the fifth day of treatment. Although the reasons for the progressively decreasing effects are not clear, two hypotheses could be offered to explain this finding. (1) Progressive tolerance to the drug is developed soon after the first day of oral administration. The decrease in the diuretic action would then be due to a progressive diminution in the over-all effects of the drugs. Against this hypothesis speaks the fact that doubling of the dose in the same patient did not materially increase its effects. Furthermore, it is difficult to visualize the development of a tolerance to a drug immediately after the first day of treatment in patients who had never taken it before. (2) The second hypothesis is that in the patients to whom the drug was given, the stores of electrolytes and water available for excretion are somewhat limited. Under these circumstances, a large portion of these stores is excreted on the first day, but as the available stores become meager, the excretion of further quantities of electrolytes become less and less. In favor of this hypothesis is the fact that a sharper decline in electrolyte excretion after the first day occurred in the patients who had less edema than in those who had severe edema. Also, the patients who had a greater diminution in diuretic response had also a significant fall in plasma electrolytes during the periods of drug administration which indicates a certain depletion of body electrolytes. Those who had a greater degree of edema had less or no fall in their plasma electrolytes.

The response to the drug of the patient who was studied while on a low-salt diet also favors the hypothesis of limited stores available for excretion as being one of the reasons for the progressively decreasing effects of the drug. In this patient, sodium and chloride excretion increased for only one day and, thereafter, fell to or below control values despite continuation of therapy while potassium excretion continued at a high rate (Fig. 5). Also, in this patient, plasma electrolytes fell considerably, indicating a severe depletion of her body electrolytes. All these factors seem to indicate that the drug may have greater and more persistent effect whenever the body fluids are expanded. In fact, this is true in the case of most of the diuretic agents. Nevertheless, chlorothiazide seems to affect plasma electrolytes more rapidly than other diuretics in the presence of a low-sodium intake and in the absence of a large degree of edema. Under these circumstances the high excretion of potassium might lead to a severe hypokalemia. Although in none of our patients symptoms of electrolyte deficiency were observed, this does not mean that they may not occur and affect the patient adversely if not properly corrected.

In the present set of experiments, the drug seems to have a flat dose-response relationship curve when given orally since the effects of 2,000 mg. dose were not significantly different from those of 500 mg. However, it should be emphasized that the conditions of the experiment might have obscured the presence of a certain dose-response relationship. In the 2 patients who were studied during 2 periods of chlorothiazide treatment (one is represented in Fig. 1) the stores of water and electrolytes might have been somewhat depleted by the first course of treatment. Under these circumstances, doubling of the dose after a short drug-free interval would not materially increase the diuretic effects. The same problems of difference in available stores of water and electrolytes apply to the different patients who were studied with different doses of the drugs. Moyer and his co-workers[3] have observed increasing responses with increasing doses below 1 mg. per kilogram in dogs. Their data, however, show that increasing the dose from 1 mg. to 10 mg. per kilogram results in only a slight increase in response. At any rate, it appears that satisfactory results with minimal side effects can be achieved with any dose which falls between 500 and 2,000 mg. although variation in response to a given dose might occur from one patient to another.

The absence of any significant action on the blood pressure is in disagreement with some reports on the effects of this drug in hypertensive conditions.[9, 10] A possible reason is that in our studies, chlorothiazide was given alone and the period of observation was relatively short. Those who have claimed antihypertensive action have usually used the drug with other vasodepressor agents and for a longer period of time. Studies are now in progress to assess the action of prolonged treatment with chlorothiazide alone or in combination with vasodepressor drugs on the hypertension of toxemia of pregnancy.

The retention of water and electrolytes which invariably occurs after cessation of therapy with chlorothiazide is peculiar to all diuretic agents. It seems to represent a homeostatic compensatory mechanism on the part of the organism to restore a part of the electrolytes lost during diuresis. The mechanisms involved in this action are, however, obscure. Nevertheless, this homeostatic action should not preclude the use of this agent to remove edema. In our study the recovery period lasted for 3 to 4 days and the quantity of electrolytes retained in this period was significantly less than the increment excreted during therapy. Whether longer recovery periods would have resulted in a greater electrolyte retention cannot be stated at this time. However, it is possible, by adjusting the drug-free intervals, to keep the patient in a negative water and electrolyte balance even though some of the electrolytes lost during treatment may be recovered after cessation of therapy. Furthermore, it is usually possible to reinitiate the diuresis when administration of the drug is reinstituted.

SUMMARY AND CONCLUSIONS

1. Studies on the diuretic effects of chlorothiazide were carried out on 15 patients with toxemia of pregnancy.

2. Orally, in doses varying from 500 mg. to 2,000 mg. per day, the drug increased markedly the excretion of Na, Cl and to a lesser extent K, and water. Plasma electrolytes fell in 4 cases and remained unchanged in 6 others. When chlorothiazide was given intravenously, electrolyte excretion increased strikingly without significant changes in renal hemodynamics.

3. Chlorothiazide seems to inhibit renal reabsorption of Na and Cl selectively, although when administered intravenously it gives evidence of renal carbonic anhydrase inhibition.

4. The diuretic effects of the drug were more striking and more persistent in the patients who had a greater degree of edema and in the patients on a liberal salt intake. In these cases, depletion of body electrolytes did not occur.

5. In the doses given orally or intravenously, the drug did not seem to have a true hypotensive action.

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STUDIES ON THE RENAL CONCENTRATING MECHANISM

I. Role of Vasopressin

Lawrence G. Raisz, M.D.,* William F. McNeely, M.D., and
Lawrence Saxon, M.D.**
Boston, Mass.

DURING dehydration the kidney conserves water by the excretion of hypertonic urine. At relatively low rates of solute excretion the renal concentrating capacity is considered to be limited by the osmotic urine/plasma (U/P) ratio, which in normal man attains a maximal value of about 4.2.[1] This ability to excrete a concentrated urine has been considered to require the action of antidiuretic hormone (ADH). Available data concerning the reproducibility of the maximum osmotic U/P ratio in the same subject or its constancy during prolonged dehydration are limited. The present study was therefore undertaken in order to determine the variability of the maximum osmotic U/P ratio during moderate dehydration in man and to assess the possible relationship of ADH to observed variations in this function. Osmotic U/P ratios have been measured during dehydration and compared with values observed during the administration of varying doses of vasopressin,† a purified extract of posterior pituitary gland which is presumed to act in the same manner as ADH. The renal responses to vasopressin in moderately dehydrated subjects have also been compared with those elicited in the same subjects after water loading.

CHEMICAL METHODS AND CALCULATIONS

Sodium, chloride, potassium, urea, and creatinine were determined in blood and urine by methods previously reported from this laboratory.[2] Total solute concentration (mOsm./Kg. of serum or urine water, i.e., osmolal concentration) of serum and urine was determined cryoscopically using a Fiske freezing point apparatus (Advanced Instruments, Needham, Mass.). Measurements of osmolality were reproducible to within 2 per cent. Glomerular filtration rate was estimated by the clearances of creatinine (C_{Cr}) and of inulin (C_{In}).[3] Urinary solute concentration and excretion are expressed as the osmotic U/P ratio and the osmolal clearance ($C_{osm} = \frac{U_{osm}}{P_{osm}}V$). Free

From the Medical Service and Research Laboratory, Boston Veterans Administration Hospital, the Departments of Medicine, Boston University School of Medicine and Tufts University School of Medicine, Boston, Mass.

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*Present address: Veterans Administration Hospital, Syracuse, N. Y.

**Postdoctoral Research Fellow, National Heart Institute, United States Public Health Service, Bethesda, Md.

†A single lot of aqueous Pitressin was used: Lot M-1761-H, Parke, Davis & Company. This lot was derived from a mixture of hog and beef pituitaries. Material was kept in a refrigerator and a fresh ampule was used in each experiment. Three bio-assays (U.S.P. XV), done at different times on this material, yielded an average potency of 17.2 pressor units per milliliter (range 16.5 to 18.7 units) and indicated that there was no decrease in potency during the study. The assays were performed by Mr. L. C. Rowe of Parke, Davis & Company at the request of Dr. D. A. McGinty, to both of whom the authors wish to express their gratitude.

TABLE I. STUDIES DURING PROLONGED WATER DEPRIVATION

SUBJECT AND AGE	DURATION OF DEPRIVATION (HOURS)	NO. OF PERIODS*	OSMOTIC U/P RATIO			OSMOLAL CLEARANCE* (ML./MIN.)		CREATININE CLEARANCE* (ML./MIN.)	
			AT 18 HOURS	AFTER 18 HOURS					
				MEAN	RANGE	MEAN	RANGE	MEAN	RANGE
L. S. 29	62	19	4.3	4.4	4.1-4.6	1.4	1.1-2.1	138	115-159
L. R. 30	48	10	4.0	3.9	3.6-4.1	1.0	0.7-1.4	121	105-136
W. M. 29	32	7	4.1	3.8	3.2-4.1	1.2	0.9-1.5	113	97-122
R. W.† 33	42	9	4.1	4.1	3.7-4.4	1.7	1.3-2.1	161	141-179
A. B.† 56	39	8	3.2	3.6	3.2-3.7	2.1	1.4-2.9	130	96-154

*Includes collection at 18 hours and subsequent periods.
†Patients with skin disease kept at bed rest on constant diets.

TABLE II. EFFECTS OF VASOPRESSIN IN DEHYDRATED SUBJECTS

SUBJECT, AGE, AND SURFACE AREA	DATE	VASOPRESSIN DOSE (MU./HR.)	OSMOTIC U/P RATIO		OSMOLAL CLEARANCE (ML./MIN.)		CREATININE CLEARANCE (ML./MIN.)		SERUM TOT. SOL. MOSM./KG. H_2O
			BEFORE	AFTER	BEFORE	AFTER	BEFORE	AFTER	
L. R. 30 1.81 M.²	9/28/54	17	3.99	3.87	1.4	2.1	104	122	283
	6/ 2/55	26	4.04	3.66	1.9	2.3	199	195	288
	10/12/54	40	3.93	3.79	1.3	*	122	*	287
	10/21/54	101	3.82	3.44	2.1	3.1	138	158	284
	2/16/54	*5,000*†	4.22	3.87	1.5	1.5	151	148	294
	2/ 9/54	*6,000*	3.77	3.77	2.1	2.9	153	160	296
	3/14/54	*10,000*	4.08	3.91	1.4	1.4	110	124	298
	3/15/54	*10,000*	4.20	3.86	1.3	1.3	111	116	299
W. M. 29 2.02 M.²	5/ 9/55	26	3.71	3.86	1.1	1.3	112	135	291
	10/ 7/54	50	3.90	3.72	1.9	2.1	124	128	286
	9/21/54	83	3.61	3.59	2.3	2.9	139	135	290
L. S. 29 1.92 M.²	5/26/55	13	4.21	4.17	2.4	1.3	147	159	293
	5/19/55	18	4.13	3.96	2.2	1.9	155	135	285
	11/14/54	*2,000*	4.33	3.84	1.3	2.2	136	138	288

*Incomplete urine collection.
†Doses in italics given by subcutaneous injection.

water clearance (C_{H_2O}) was calculated from the rate of urine flow and the osmolal clearance as follows: $C_{H_2O} = V - C_{osm}$. A positive value is thus a measure of the excretion of solute-free water, while a negative value indicates the volume of water which would be required to dilute a given hypertonic urine to a concentration iso-osmotic with the plasma.

1. STUDIES DURING PROLONGED DEHYDRATION

Methods.—Three normal men (L. S., L. R., and W. M.) and two hospitalized ambulatory patients (R. H. and A. B.) with chronic skin conditions were studied while deprived of fluid intake for 32 to 62 hours. The 3 normal subjects continued their usual activities and ate dry diets of varied protein and salt content. Urine was collected at varying intervals. In order to minimize the possible influences of activity and dietary intake the 2 patients remained in bed throughout the period of study, rising only to void at intervals of approximately 3 hours, and ate a constant dry diet divided into roughly equal portions at 7 A.M., 12 noon, 6 P.M., and 11 P.M. The diet provided a minimum of 40 Gm. of protein and contained less than 500 Gm. of preformed water per day.

RESULTS

Although the degree of dehydration as measured by serum osmolality increased steadily throughout the period of ingestion of the dry diet in all subjects, there was no parallel steady increase in the osmotic U/P ratios of the normal subjects after the first 18 hours (Table I). In the 4 young subjects, whose ages ranged from 29 to 33 years, osmotic U/P ratios of greater than 4.0 were achieved with values in individual periods varying from 3.2 to 4.6. The older subject (A. B., aged 56) did not achieve his maximum urinary concentration until the period 21 to 24 hours after water deprivation was begun when osmotic U/P ratio was 3.6. Thereafter the U/P ratio remained fairly constant despite fluctuations in osmolal clearance and creatinine clearance. However, these fluctuations may have been due to errors in emptying the bladder. The study of R. H. is charted in Fig. 1. The lowest values for U/P ratio were obtained between the hours of 3 and 9 A.M. of the final day of dehydration in this subject, while the highest values occurred at this time in other subjects. There was no consistent correlation between the fluctuations in osmotic U/P ratio and the changes observed in creatinine or osmolal clearances. Both clearances were lower during the last 12 hours in R. H., but the other young subjects showed only small and irregular variations. The variation in osmotic U/P ratio in these data could not be related to the proportions of total urine osmolality contributed by Na, K, Cl, or urea.

2. STUDIES OF THE EFFECTS OF VASOPRESSIN IN DEHYDRATED SUBJECTS

Methods.—Fourteen studies were performed on 3 normal men who had abstained from fluid intake for 18 to 22 hours. All studies on Subjects L. R. and W. M. were performed in the morning and those on Subject L. S. in the afternoon. After collection of control urine samples, venous blood samples were obtained and vasopressin was administered in various doses either by subcutaneous injection or by constant intravenous infusion, the latter being given in isotonic saline infused by means of a Bowman pump at a rate of 0.6 ml. per minute. A 30- to 90-minute urine collection was then obtained to assess any detectable effects of the administered vasopressin.

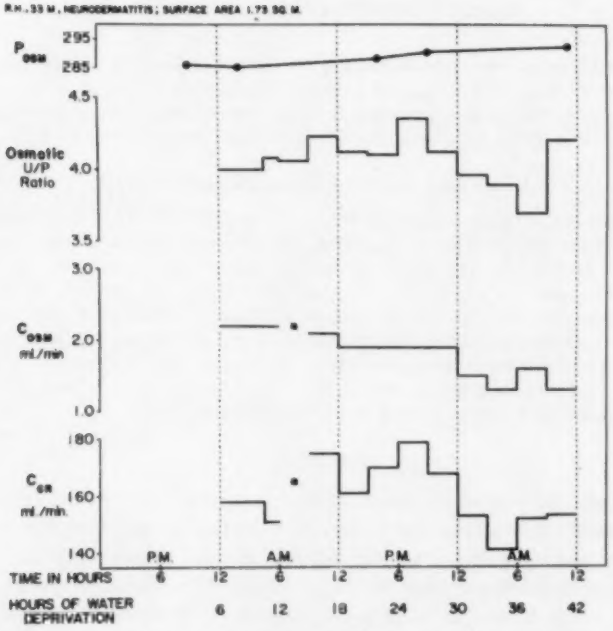

Fig. 1.—Effects of prolonged dehydration in a hospitalized subject without renal disease. Serum solute concentration (P_{Osm}, mOsm./Kg. H_2O), osmotic U/P ratio, osmoial clearance (C_{Osm}), and endogenous creatinine clearance (C_{Cr}) are shown. Water deprivation was begun at 6 P.M. *Part of urine specimen discarded.

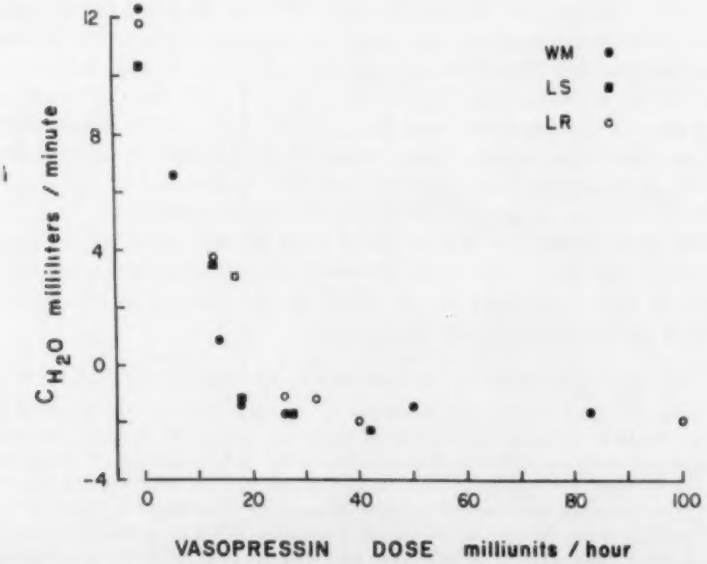

Fig. 2.—Free water clearance (C_{H_2O}) versus vasopressin dose in hydrated normal subjects.

RESULTS

The maximum osmotic U/P ratio achieved after 18 to 22 hours of water deprivation varied between 3.6 and 4.3 in these studies (Table II). The variations could not be correlated with variations in serum solute concentration. After this period of dehydration vasopressin administration over a wide range of doses did not appear further to increase the osmotic U/P ratio. Actually two of the 3 subjects showed a consistent small decrease in osmotic U/P ratio after vasopressin administration. In most instances this decrease was accompanied by a rise in osmolal clearance. Creatinine clearance showed no consistent changes after vasopressin administration.

3. EFFECTS OF VASOPRESSIN IN REHYDRATED SUBJECTS

Methods.—The effect of vasopressin during rehydration was examined in the same subjects and usually as a continuation of one of the experiments described in Section 2. After 18 to 22 hours of dehydration an infusion was begun containing vasopressin and rehydration was accomplished by administration of an oral water load of 20 ml. per kilogram of body weight while vasopressin infusion was continued.*

RESULTS

The data given in Table III are average values for 2 to 4 collection periods after the water load had been given. Fig. 2 illustrates the relationship between C_{H_2O} and vasopressin dose. When no vasopressin was given C_{H_2O} was 10.3 to 12.3 ml. per minute. During the infusion of 6 to 18 mu. per hour of vasopressin C_{H_2O} was inversely proportional to the vasopressin dose. In Subjects W. M. and L. S., C_{H_2O} was negative when vasopressin was infused at the rate of 18 mu. per hour, but osmotic U/P ratios were only 1.6. During the infusion of 25 to 27 mu. per hour mean osmotic U/P ratios were 3.37 and 3.83. In W. M. the osmotic U/P ratio was still higher during the infusion of 50 mu. per hour; a dose of 83 mu. per hour had no greater effect. Although in Subject L. S. the mean osmotic U/P ratio was the same during infusions of 27 and 42 mu. per hour, this may have been related to the difference in solute excretion between the two experiments, since during individual periods with similar rates of solute excretion U/P ratio was slightly higher at the higher dose. The dose-response curve of Subject L. R. differed somewhat in that slightly larger doses of vasopressin were required to produce a given osmotic U/P ratio.† Thus, the ratio was only 3.15 when the dose was 42 mu. per hour, but reached 3.80 when the dose was 101 mu. per hour. There was no further increase when

*West, Traeger, and Kaplan[4] have reported that when vasopressin infusions were begun during a water diuresis, osmotic U/P ratios reached only about 3.0 even after 3 hours of infusion of large doses. In preliminary studies we found that when a vasopressin infusion (100 mu. per hour) was begun during an established water diuresis, osmotic U/P ratio gradually increased for 4 to 5 hours and finally reached values of 3.8 to 4.0. This slow response was not accelerated by increasing the dose or giving a priming dose at the beginning of the infusion. On the other hand, when vasopressin was administered during a combined water and osmotic (mannitol) diuresis, the maximum osmotic U/P ratio of 2.2 to 2.6 was achieved within 2 hours. We, therefore, believe that the slow increase in osmotic U/P ratio at low rates of solute excretion may be due to slow equilibration in the dead space of the urinary tract, although delay in the response of the renal tubular concentrating mechanism to vasopressin cannot be ruled out. Administration of vasopressin before water loading obviates this difficulty and thus was employed in the present study.

†This subject had received repeated subcutaneous and intravenous injections of vasopressin several years prior to the present study and may conceivably have been slightly less sensitive to its action.

TABLE III. EFFECTS OF VASOPRESSIN IN HYDRATED SUBJECTS

SUBJECT	AND DATE	VASOPRESSIN DOSE (MU./HOUR)	OSMOTIC U/P RATIO	V (ML./MIN.)	C_{H_2O} (ML./MIN.)	C_{osm} (ML./MIN.)	C_{in} (ML./MIN.)	C_{cr} (ML./MIN.)	$\frac{C_{cr}}{C_{in}}$ (ML./MIN.)	SERUM TOT. SOL. (MOSM./KG. H_2O)
L. R.	10/ 5/55	0	0.22	15.1	11.8	3.3	115	152	1.32	273
	11/15/55	13	0.45	6.9	3.8	3.1	124	148	1.20	272
	9/28/54*	17	0.49	6.1	3.1	3.0	126	162	1.29	278
	1/20/56	26	1.63	1.6	−1.1	2.7	117	150	1.27	274
	11/ 8/55	32	1.74	1.6	−1.2	2.8	118	146	1.24	271
	10/12/55	40	3.15	0.9	−2.0	2.9	123	172	1.40	276
	10/21/54	101	3.80	0.7	−2.0	2.7	126	162	1.29	273
	2/ 9/54*	5,000 (S.C.)	3.72	1.0	−2.8	3.8	†	178	--	281
W. M.	9/10/55	0	0.23	16.0	12.3	3.7	110	137	1.25	278
	10/12/55	6	0.30	9.4	6.5	2.9	103	141	1,37	281
	9/ 9/55	14	0.82	4.6	0.9	3.7	114	146	1.28	275
	7/14/55	18	1.64	2.3	−1.4	3.7	113	145	1.28	278
	5/ 9/55	26	3.37	0.8	−1.7	2.5	†	135	--	275
	10/ 7/54	50	4.07	0.4	−1.5	1.9	116	139	1.20	276
	9/21/54	83	3.73	0.6	−1.7	2.3	†	131	--	279
L. S.	9/22/55	0	0.27	13.4	10.3	3.1	112	150	1.34	280
	10/22/55	13	0.55	7.7	3.5	4.2	116	159	1.37	276
	5/26/55	18	1.66	2.0	−1.2	3.2	123	154	1.26	282
	5/19/55	27	3.83	0.6	−1.7	2.3	†	165	--	278
	4/ 6/55	42	3.80	0.8	−2.3	3.1	118	154	1.27	282

*One period only. Other data are averages of 2 to 4 collection periods.
†Inulin clearance not measured.

5,000 mu. per hour was administered subcutaneously. The values obtained are considered to represent the effects of exogenous vasopressin alone since sustained water diuresis developed when no vasopressin was given, and since in one experiment (L. R., 10/12/55) the ingestion of 120 ml. of 100 proof whiskey did not alter osmotic U/P ratio while vasopressin was being infused at a rate of 40 mu. per hour. Vasopressin administration did not alter inulin or creatinine clearance or the creatinine/inulin clearance ratio.

DISCUSSION

In the present study the osmotic U/P ratios attained after 18 hours of water deprivation were "maximal" only in the sense that osmotic U/P ratio did not increase progressively with continued water deprivation. However, considerable variation in osmotic U/P ratio was observed not only during prolonged water deprivation but also with successive tests of the effects of 18 hours of water deprivation in the same subject. These variations did not follow the pattern of a diurnal rhythm and did not appear to depend on variation in the plasma solute concentration. A fall in urine osmolality and rise in rate of flow associated with augmentation of solute excretion in the hydropenic state has been studied extensively using large osmotic loads.[5-7] The possibility that maximal osmotic U/P ratio at lower flows might also depend on the rate of solute excretion was suggested by the inverse relationship between these functions sometimes observed in dehydrated subjects given large doses of vasopressin. More detailed observations during dehydration indicate that an acute increase in C_{osm} over the range of 1 to 5 ml. per minute regularly leads to a decrease in osmotic U/P ratio.[8] In more prolonged studies variations in osmotic U/P ratio can be due to other factors such as the dietary protein intake.[9]

The possibility that variations in endogenous ADH production are responsible for the variations in osmotic U/P ratio observed after 18 to 22 hours of water deprivation seems unlikely since vasopressin administration did not produce a rise in osmotic U/P ratio at this time. When vasopressin administration was continued in adequate doses, rehydration with an oral water load did not lower osmotic U/P ratio in our subjects. West and associates[4] and Jones and deWardener[10] have noted that when previously dehydrated subjects were receiving vasopressin continuously the administration of a water load did not lower urine solute concentration. However, these authors and others[11, 12] have reported that prehydrated subjects receiving large doses of vasopressin do not excrete as concentrated a urine as do dehydrated subjects. These differences were greatest in subjects who were given excess water for several days.[12] In preliminary studies we found that when vasopressin infusion was begun in subjects who had just been rehydrated and were undergoing a water diuresis U/P ratios of 3.8 to 4.0 were ultimately achieved but only after 4 to 5 hours of vasopressin administration. Maximal concentration of the urine may require the prolonged continuous presence of endogenous ADH, or may depend on some effect of dehydration which is not mediated through the secretion of ADH. It is also possible that the neurohypophyseal secretion of man has an action which differs from that of vasopressin.

The dose-response curve obtained when vasopressin is administered in low doses to water loaded subjects has been studied in detail by Hollander, Williams, Fordham, and Welt.[13] These authors present several equations describing the relationship between vasopressin dose and the response, ΔC_{H_2O}, the latter function being the magnitude of the fall in free water clearance from the value observed during maximal water diuresis. The present observations at low doses of vasopressin can be fitted to similar equations. However, the equations obtained from observations at low doses do not describe the response to high doses of vasopressin. When C_{H_2O} becomes negative with large doses there is a change in the slope of the dose-response curve which is consistent with the view that some, but not all, of the tubules are already saturated with vasopressin. Further increases in dose lead to small increments in water reabsorption and osmotic U/P ratio until a "maximal" dose is ultimately reached, presumably when all of the tubules are saturated. These findings are in agreement with the work of Lauson[14] who found that vasopressin in doses of 50 mu. per hour produced a maximal effect.

SUMMARY

The ability of the kidney to form a concentrated urine at low rates of solute excretion as measured by osmotic U/P ratio was studied during moderate dehydration in 5 essentially normal men. After 18 hours of water deprivation the osmotic U/P ratios showed irregular variations which were not correlated with the serum total solute concentration and did not take the form of a diurnal rhythm. The osmotic U/P ratio was measured after 18 to 22 hours of water deprivation on repeated occasions in 3 normal young men and found to vary between 3.6 and 4.3. This value was not increased by administration of vasopressin.

The relationship between vasopressin dose, free water clearance, and osmotic U/P ratio was examined in the same subjects after hydration. Although free water clearance became negative when vasopressin was given in doses of 18 to 26 mu. per hour, the highest osmotic U/P ratios were not achieved until 42 to 100 mu. per hour was infused. U/P ratios were then 3.7 to 4.1, values similar to those found in the same subjects during moderate dehydration.

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SIZE OF MUMPS SKIN TEST REACTIONS

DANIEL N. TUCKER, B.S., AND JOHN R. OVERMAN, M.D.
DURHAM, N. C.

SEVERAL studies have demonstrated the usefulness of mumps skin testing since the reaction was first described by Enders in 1943.[1] The most comprehensive experiments are those of Henle and associates[2] who studied the skin test reaction in relation to history of mumps, complement fixation and hemagglutination-inhibition antibody titers, and resistance to the development of clinical mumps. In addition, the effect of skin test antigen on circulating mumps antibody levels was described. Other papers by Cabasso,[3] Muntz,[4] and Florman[5] described mumps skin testing under various clinical situations.

Only the original report of Enders, however, gave detailed data on the size of reactions obtained as correlated with antibody studies on the subjects tested. Cabasso reported the size of each reaction he obtained as an average of the 24- and 48-hour readings but this study did not include serum antibody determinations so that evaluation of the significance of the size of the reactions is difficult. Enders suggested 15 mm. or larger as the minimum positive reaction and the studies of Henle followed this criterion. Florman,[5] however, designated 10 mm. as the minimum positive reaction. Thus, the criteria of size of the mumps skin test reactions are not well established, particularly since Enders' study used monkey parotid antigen whereas all present commercial antigen is prepared from egg-grown virus. The present paper reports the size of the skin reactions of a group of young adults correlated with history and serum mumps hemagglutination-inhibition and neutralizing antibody titers.

MATERIALS AND METHODS

Skin Tests.—Mumps skin test antigen and control material was generously supplied by Lederle Laboratories. The subjects were sophomore medical students at Duke University, all in good health and none of whom had had mumps more recently than 10 years prior to the present study nor had they knowingly been recently exposed to mumps. There has been no large epidemic of mumps in North Carolina in the past several years. The procedure for testing recommended by Lederle was followed. Thus, 0.1 ml. of control and antigen was injected intradermally and skin reactions were measured carefully at 24 and 48 hours following the injections.

Serum Antibody Studies.—The mumps virus antigen was kindly supplied by Dr. Victor Cabasso of Lederle Laboratories as lyophilizeed mumps infected allantoic fluid. It has been maintained in this laboratory by passage in 7-day-old embryonated eggs. Serum to

From the Department of Bacteriology, Duke University School of Medicine, Durham, N. C.
Address reprint requests to Dr. John R. Overman.
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be tested for mumps antibody was diluted 1:10 in normal saline and then inactivated at 56° C. for 30 minutes. In the test 0.2 ml. of serum dilutions were mixed with 0.2 ml. mumps virus allantoic fluid diluted to certain 8 hemagglutinating units as described previously.[6] This test as performed in this laboratory gives no false positive reactions. In some instances mumps neutralizing antibody was determined and the method used has been previously described in detail.[6]

RESULTS

The results obtained are summarized in Table I. It is apparent that a good deal of the specificity and value of the skin test depends on the criteria used for a positive test. If the minimum criterion is that described by Enders (as 15 mm.) then the test is quite specific and relatively reliable. For example, none of the individuals who had not had mumps on the basis of serum antibody studies gave a positive skin test reaction of 15 mm. or greater. There were in this series, therefore, no false positives. The skin test, however, failed to correlate with antibody studies in 7 of 28, giving 25 per cent false negative reactions. To determine more definitively whether these patients had actually had mumps, virus neutralization tests were performed on all these sera and all showed neutralizing antibody in a serum dilution of 1:10.

TABLE I. SIZE OF MUMPS SKIN TEST REACTIONS

MUMPS ANTI-HEMAGGLUTINATION ANTIBODY	HISTORY OF MUMPS	CRITERIA OF MINIMUM SKIN REACTIONS (MM.)	SKIN TESTS POSITIVE	SKIN TESTS NEGATIVE	POSITIVE CONTROL
		15 mm.	14	2*	1
Positive 28	Positive 17	10	14	2*	1
		5	15	1	1
		15	5	5*	1
	Negative 11	10	5	2*	4
		5	7	0	4
		15	0	5	1
Negative 10	Positive 6	10	2	3	1
		5	4	0	2
		15	0	4	0
	Negative 4	10	3	1	0
		5	4	0	0

*Mumps neutralizing antibody titrations performed on these sera (see text).

It is also obvious from the data in Table I that reduction of the criteria of the test to less than 15 mm. increases the number of false positive reactions without increasing the number of positive reactions in persons who had had mumps. Thus, the 25 per cent false negatives were not substantially changed by reducing the minimum criterion to 10 mm.

Some of the individual reactions are particularly worth noting. Using reactions of 15 mm. or larger as the minimum criterion for a positive test, most positive reactors at 24 hours became more strongly positive at 48 hours. Thus of all those 17 designated finally as positive, 16 increased the size of the reaction, and only one became significantly smaller from 48 hours after the test. It is also of interest that at 48 hours the nonspecific control reactions tended to decrease in size so that of 10 controls at 24 hours 9 had decreased in size to a significant degree by 48 hours. These findings would suggest that the 48-hour interval is most reliable for reading of the test.

An analysis of some reactions showed that failure to read the test at both 24 and 48 hours would allow some positive reactors to be missed. One individual had a 16 mm. reaction at 24 hours which decreased to 4 mm. at 48. The control was entirely negative at both intervals. Conversely, another subject's reaction was clearly negative at 24 hours and clearly positive at 48, also with entirely negative controls.

DISCUSSION

These experiments suggest that the minimum criteria used in the mumps skin test be reactions of 15 mm. or greater for a positive test. If this size is adopted the reaction is then quite specific for persons who, on the basis of antibody studies, have had mumps and will not include false positive reactions. It must be noted, however, that 25 per cent of persons who had had mumps as indicated by both serum hemagglutination-inhibition and virus neutralizing antibody will fail to react. Most of these in this study came from the group which by history must have had an inapparent infection. Since all of the subjects had an interval of 10 or more years between the present studies and their mumps infection, it may be that these individuals simply lost their skin sensitivity. It may be, however, that the less severe clinical infections tend to result in a lesser degree of skin sensitization.

SUMMARY

Mumps virus skin test reactions were studied in 38 individuals and correlated with serum mumps antibody levels. The results indicate that positive reactions must be characterized by an area of erythema 15 mm. or greater in size. In persons many years convalescent from mumps, 25 per cent may fail to react to the skin test antigen even though they maintain significant mumps serum antibody.

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OBSERVATIONS ON HEMOPHILIA, PARAHEMOPHILIA, AND COEXISTENT HEMOPHILIA AND PARAHEMOPHILIA

Alterations in the Platelets and the Thromboplastin Generation Test

Richard H. Seibert, M.D., Alvin Margolius, Jr., M.D., and
Oscar D. Ratnoff, M.D.
Cleveland, Ohio

ALTHOUGH its existence stretches credulity, the combination of hemophilia and parahemophilia in the same patient has been reported. Oeri and his associates[1] and Iversen and Bastrup-Madsen[2] have described 3 individuals with life-long hemorrhagic states whose plasmas were deficient in "classic" antihemophilic factor and proaccelerin. A fourth patient, studied recently at University Hospitals of Cleveland, has provided the opportunity to make some observations on the genetic nature of this curious disease, as well as to extend studies of the platelets and of the thromboplastin generation test in classic hemophilia and parahemophilia. These studies have corroborated the observations of Lewis,[3] Hjort and his associates,[4] and Mann[5] that the platelets of patients with parahemophilia are deficient in proaccelerin-like activity and those of patients with classic hemophilia are deficient in antihemophilic activity. Moreover, studies of the thromboplastin generation test confirm the view that proaccelerin is needed for the optimal generation of clot-promoting activity in the first stage of this procedure.

CASE REPORTS

Case 1.—N. S. (University Hospitals No. 678036), a 13-year-old boy, was admitted to the Babies' and Children's Hospital on July 21, 1955, subsequent to hemorrhage into the right thigh. While in the hospital he had a massive hematemesis; transfusion with freshly frozen plasma and fresh whole blood was followed by a cessation of bleeding. The patient's earliest episode of bleeding was hemorrhage after tonsillectomy at the age of 5. Subsequently he had frequent epistaxes and episodes of bleeding into muscles and subcutaneous tissues, but no recognized hemarthroses.

Only the maternal branch of the family was available for study. The maternal great-grandmother and great-grandfather were first cousins. None of the maternal family had overt bleeding difficulties.

Studies of the coagulative mechanisms are tabulated in Table I.

Case 2.—B. L., a 29-year-old male chemist, was studied in December, 1956, at a time when he was asymptomatic. Hemorrhagic phenomena were first recognized when he was 6 years old. Subsequently he had hemarthroses of knees and ankles, spontaneous ecchymoses,

From the Department of Medicine, the Western Reserve University School of Medicine and University Hospitals, Cleveland, Ohio.

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gingival bleeding, epistaxes, and severe hemorrhages following injury of a wrist, circumcision, and dental extractions. Treatment on at least two occasions included blood transfusion. No residual deformities were evident on physical examination.

The patient's parents both came from a Yugoslavian town with a population of about 1,500, but there was no recognized consanguinity. His mother always had excessive menstrual bleeding. As a child she had had repeated nosebleeds. However, she was unaware of spontaneous ecchymoses or excessive bleeding subsequent to surgery, dental extraction, injuries, or childbirth. The patient's father, now deceased, had no history of a bleeding tendency, nor does his maternal half-brother.

Studies of the coagulative mechanisms are tabulated in Table I.

MATERIALS AND METHODS

Nomenclature.—The terms classic hemophilia or hemophilia will refer to the hereditary disorder characterized by a deficiency of antihemophilic factor. Parahemophilia will be used to describe the congenital deficiency of proaccelerin (factor V, plasma accelerator globulin). Thromboplastin will be used in the generic sense for any active principle which, in the presence of proaccelerin, serum factors, and calcium, converts prothrombin to thrombin; thus thromboplastin is present in animal tissues and may evolve in shed blood during the process of coagulation.

Materials.—Blood was drawn from the antecubital veins of human subjects, using No. 18 gauge needles coated with tris(2-hydroxyethyldodecyl)ammonium chloride (Monocote, Armour) and syringes coated with silicone (G.E. Dri-Film).

Oxalated and citrated plasma, aluminum hydroxide-adsorbed plasma, platelets, and platelet-poor plasma were prepared by previously described techniques.[6] Serum was prepared by incubating whole blood in Pyrex tubes at 37° C. for a minimum of 2 hours and separating the serum by centrifugation. The serum was stored at −25° C. until used.

Prothrombin-deficient bovine plasma was prepared by the method of Owren.[7]

Crude antihemophilic factor was prepared from aluminum hydroxide-adsorbed citrated plasma by precipitating the fraction insoluble at one-third saturation with neutralized ammonium sulfate. The precipitate was separated by centrifugation, dissolved in water, and reprecipitated at one-third saturation with ammonium sulfate. The precipitate was then dialyzed against barbital-saline solution at 4° C.

Crude proaccelerin was prepared in the same way from the fraction soluble at one-third saturation but insoluble at one-half saturation with ammonium sulfate.

Commercial rabbit brain thromboplastin (Difco), human brain thromboplastin (Cappell), and Russell's viper venom (Burroughs Wellcome) were suspended by methods previously described.[8]

Plasma or serum deficient in antihemophilic factor, Christmas factor (plasma thromboplastin component), Hageman factor, and pro-SPCA were obtained from patients with congenital deficiencies of these factors.

Crude cephalin was prepared from desiccated rabbit brain thromboplastin (Difco) by the method of Bell and Alton.[9]

Crystalline trypsin (Worthington) was dissolved in a concentration of 1 mg. per milliliter of buffer and shaken for 10 minutes with an Amberlite IRC 50 resin, in the sodium cycle at pH 7.7, in order to remove magnesium sulfate.

Purified staphylocoagulase* was dissolved in a concentration of 0.2 mg. per milliliter of buffer.

All tests were performed in Pyrex tubes (internal diameter, 8 mm.) unless otherwise noted. Silicone-coated tubes to measure the clotting time of whole blood were prepared by rinsing clean, new Pyrex tubes with 0.1 per cent solution of G.E. Dri-Film in petroleum ether. Excess silicone was removed with copious amounts of distilled water and the tubes were then dried in an oven at 150° C.

*Dr. M. Tager of Emory University kindly provided the purified staphylocoagulase.

The preparation of buffered sodium chloride solution, composed of 0.025 M barbital and 0.125 M sodium chloride at pH 7.5 was described previously.[8] All glassware was washed with chromic acid and rinsed with distilled water. Dialyses were performed in Visking No-Jax cellophane sausage casings one-fourth inch in diameter.

Methods.—The methods used for the determination of the clotting time of whole blood in glass or silicone tubes,[10] recalcified clotting time of plasma,[10] thrombin time,[10] prothrombin time[11] prothrombic activity of serum,[6] clot retraction,[12] direct platelet count,[13] and plasma fibrinogen concentration[14] have all been described earlier.

The thromboplastin generation test was performed as originally described by Biggs and Douglas.[15] In this test, a mixture of dilute aluminum hydroxide-adsorbed plasma, dilute serum, and platelets is calcified and incubated at 37° C. When these reagents are prepared from normal blood, this *incubation mixture* is deficient in prothrombin but is thought to contain all other clotting factors. Thromboplastin-like activity is believed to evolve in this mixture and is measured in the following manner. At intervals, an aliquot of 0.1 ml. is withdrawn and pipetted into a tube containing 0.1 ml. of platelet-deficient plasma (or *substrate*). The substrate is then immediately recalcified by the addition of 0.1 ml. of 0.025 M calcium chloride and its clotting time is measured at 37° C. In a normal incubation mixture, sufficient thromboplastin-like activity is generated within 4 to 6 minutes to clot the substrate plasma in 9 to 14 seconds. With abnormal components, the onset of "thromboplastin generation" may be delayed, the rate of its evolution slowed, and the minimal clotting time prolonged relative to the normal system.

In certain experiments, crude cephalin, Russell's viper venom, or human or rabbit brain thromboplastin was substituted for platelets in the incubation mixture.

The effect of normal plasma upon abnormal platelets was tested by incubating 2 ml. of a saline suspension of platelets with 9 ml. of normal platelet-deficient plasma for one hour at 4° C. The platelets were then sedimented by centrifugation, washed 4 times with saline solution, and tested in the thromboplastin generation test.

The antihemophilic activity of plasma was estimated by determining its capacity to correct the abnormally long clotting time of plasma known to be deficient in antihemophilic factor. The plasma to be tested was diluted to the desired degree with buffer. In duplicate, 0.1 ml. each of the diluted plasma, undiluted hemophilic plasma, and 0.025 M calcium chloride solution were mixed in Pyrex tubes. The clotting time was then determined at 25° C. Details of this method have been published elsewhere.[8] The Hageman factor and Christmas factor (plasma thromboplastin component) activities of plasma were tested by the same technique.

The proaccelerin activity of plasma was determined in the following manner. The plasma to be tested was adsorbed with aluminum hydroxide and diluted, when indicated, with aluminum hydroxide-adsorbed, proaccelerin-deficient plasma obtained from Patient 1. The prothrombin time of a mixture of 0.1 ml. each of the adsorbed plasma, unadsorbed proaccelerin-deficient plasma, rabbit brain thromboplastin, and 0.025 M calcium chloride solution was then measured. The effect of an unknown plasma was compared with that of a pool of several normal plasmas, similarly treated. The proaccelerin-like activity of Patient 1's plasma was assumed to be less than 1 per cent that of normal plasma.

Over-all proconvertin-like activity was tested by measuring the effect on the one-stage prothrombin time of the addition of 0.1 ml. of plasma thought to be deficient in stable serum factors. Such plasma was obtained from a patient with a greatly prolonged prothrombin time subsequent to the administration of Dicumarol. *Pro-SPCA-like* activity was determined by mixing plasma from a patient congenitally deficient in this factor* with the plasma to be tested. The diagnosis of pro-SPCA deficiency was established by the following criteria: prolonged one-stage prothrombin time corrected by normal serum but not

*Obtained through the courtesy of Dr. J. D. Battle, Jr., and Dr. J. S. Hewlett, Cleveland, Ohio.

by aluminum hydroxide-adsorbed plasma, a normal plasma clotting time in the presence of Russell's viper venom, and correction of the prothrombin time upon the addition of plasma from a patient deficient in Stuart factor.*

Plasma prothrombic activity was tested specifically by measuring the prothrombin time in the presence of 0.05 ml. of bovine barium sulfate-adsorbed oxalated plasma and 0.05 ml. of pooled normal human serum.[16] The serum and adsorbed plasma were thought to be deficient in prothrombin but supplied other factors necessary for the conversion of prothrombin to thrombin by thromboplastin. Prothrombic activity was also tested by the two-stage prothrombin time; beef serum was added to supply proaccelerin.[17]

The partial thromboplastin time was performed by the method described by Langdell, Wagner, and Brinkhous.[18] In this test, the recalcified clotting time of citrated plasma is measured in the presence of crude cephalin.

The trypsin clotting time was performed by the addition of 0.1 ml. of a solution of trypsin to 0.2 ml. of citrated plasma. The clotting time of the mixture was measured at 37° C.

The presence of staphylocoagulase-reacting factor was tested by the addition of 0.1 ml. of a solution of staphylocoagulase to 0.2 ml. of citrated plasma and measuring the clotting time at 37° C.

Tests for circulating anticoagulants were performed by previously described methods.[6, 19] The effect of the abnormal plasma on the recalcified clotting time and on the prothrombin time of normal plasma was used as a test for anticoagulants directed, respectively, against the early and middle stages of clotting.

RESULTS

1. *The Nature of the Clotting Defect in Case 1: Evidence Supporting the Diagnosis of Parahemophilia.*—The clotting time of whole blood, obtained from Patient 1, was prolonged in both glass and silicone-coated tubes (Table I). This abnormality was associated with prolongation of the one-stage prothrombin time. The defect measured by this test was corrected by the addition of normal aluminum hydroxide-adsorbed plasma; the patient's aluminum hydroxide-adsorbed plasma was without effect (Table II). Other routine tests were unremarkable except that the prothrombic activity of serum was abnormally high, evidence that the conversion of prothrombin to thrombin during clotting was impaired. These data are consistent with the diagnosis of parahemophilia, that is, a deficiency of proaccelerin. This diagnosis was corroborated by the failure of normal human serum, which is deficient in proaccelerin, to shorten the prolonged prothrombin time. Nor did normal oxalated plasma, stored at 4° C. for 5 weeks. Such plasma is also deficient in proaccelerin.

Only the patient's maternal relatives could be tested. The proaccelerin-like activity of his mother's plasma was approximately 50 per cent of that of normal plasma. The proaccelerin content of his maternal grandfather's plasma was normal; studies of a maternal uncle were equivocal.

2. *The Nature of the Clotting Defect in Case 2: Evidence Supporting the Diagnosis of Combined Hemophilia and Parahemophilia.*—The clotting time of whole blood, obtained from Patient 2, was abnormally high, and the one-stage prothrombin time was prolonged (Table I). The diagnosis of parahemophilia

*Obtained through the courtesy of Dr. J. G. Graham, Chapel Hill, N. C.

was established by the following techniques. The abnormal one-stage prothrombin time of the patient's plasma was not corrected by plasma obtained from Patient 1, nor by normal, aged oxalated plasma. The patient's aluminum hydroxide-adsorbed plasma was only a fraction as effective as normal adsorbed

TABLE I. HEMATOLOGIC STUDIES IN PATIENTS WITH PARAHEMOPHILIA AND WITH COMBINED HEMOPHILIA AND PARAHEMOPHILIA

TEST	NORMAL VALUES	PATIENT 1 (PARAHEMOPHILIA)	PATIENT 2 (COMBINED HEMOPHILIA AND PARAHEMOPHILIA)
1. Whole blood clotting time, Pyrex tubes	18-38 min.	47 min.	75 min.
2. Whole blood clotting time, silicone tubes	55-195 min.	>9 hr.	>9 hr.
3. Recalcified plasma clotting time	4-11 min.	45 min.	18 min.
4. Bleeding time (finger stick)	<10 min.	3½ min.	7½ min.
5. Tourniquet test	–	Normal	Normal
6. Platelet count (direct)	200,000-300,000	218,000	245,000
7. Clot retraction	70+ % of potential retraction	72 %	78 %
8. Thrombin time	26-34	35 sec.	30 sec.
9. Serum prothrombic activity	<25 % of plasma	75 %	31 %
10. Prothrombin time	15.3 sec. 14.4 sec.	47.1 sec. –	– 26.8 sec.
11. Plasma prothrombic activity (method of Alexander)	88-120%	100 %	75 %
12. Plasma prothrombic activity (two-stage prothrombin time)	150-280 units	–	185 units
13. Pro-SPCA-like activity	–	Normal	Normal
14. Proconvertin-like activity	–	Normal	Normal
15. Proaccelerin assay	90-110%	<1 %*	3 %
16. Partial thromboplastin time	120-130 sec.	500 sec.	
17. Antihemophilic factor assay	50-150 %	100 %	6 %
18. Christmas factor activity	–	Normal	Normal
19. Hageman factor activity	–	–	Normal
20. Staphylocoagulase-reacting factor	50 sec.	–	50 sec.
21. Trypsin clotting time	66 sec.	–	61 sec.
22. Plasma fibrinogen	200-400 mg./100 ml.	337	274
23. Test for circulating anticoagulant	–	None demonstrated	None demonstrated

*This was assumed.

TABLE II. THE EFFECT OF ALUMINUM HYDROXIDE-ADSORBED PLASMA ON THE ONE-STAGE PROTHROMBIN TIME

ADSORBED PLASMA	CLOTTING TIME* (SEC.)
Normal	20
Case 1	91
Case 2	51

*Clotting time at 37° C. of 0.1 ml. each of citrated plasma obtained from Patient 1, adsorbed plasma, rabbit brain thromboplastin, and 0.025 M calcium chloride.

TABLE III. THE EFFECT OF A CRUDE "PROACCELERIN" FRACTION ON THE ONE-STAGE PROTHROMBIN TIME

SOURCE OF "PROACCELERIN" FRACTION	CLOTTING TIME* (SEC.)
Normal subject	31
Case 2	97

*Clotting time at 37° C. of a mixture of 0.1 ml. each of citrated plasma obtained from Patient 1, crude proaccelerin, rabbit brain thromboplastin, and 0.025 M calcium chloride.

plasma in shortening the prothrombin time of parahemophilic plasma (Table II). Moreover, a crude proaccelerin fraction of the patient's plasma had no appreciable corrective effect on the prothrombin time of Patient 1's plasma (Table III). The deficiency of proaccelerin was not as profound as in Case 1; plasma obtained from Patient 2 had about 3 per cent of the corrective activity of normal plasma when added to plasma obtained from Patient 1.

A deficiency of antihemophilic factor was also present (Table IV). This defect was not complete; a crude antihemophilic fraction of plasma from Patient 2 had about 6 per cent of the antihemophilic activity of a similar fraction of normal plasma.

TABLE IV. THE ANTIHEMOPHILIC ACTIVITY OF PLASMA IN CASE 2

TEST PLASMA	CLOTTING TIME* (MIN.)
Buffer	29
Normal, diluted 1:10	10
Normal, diluted 1:40	13
Case 2, diluted 1:10	16

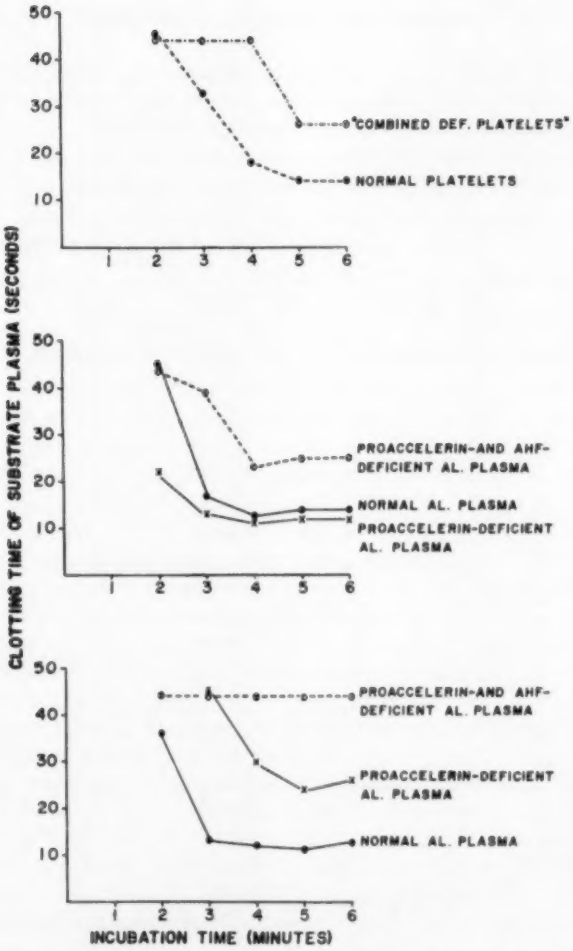
*Clotting time at 25° C. of a mixture of 0.1 ml. each of hemophilic plasma, the plasma to be tested (diluted with buffer), and 0.025 M calcium chloride solution.

Plasma from the mother of Patient 2 had approximately one-third the proaccelerin activity of normal plasma; its antihemophilic activity was normal. The proaccelerin-like and antihemophilic activities of the patient's maternal half-brother's plasma were within normal limits.

3. *The Thromboplastin Generation Test in Classic Hemophilia, Parahemophilia, and Combined Hemophilia and Parahemophilia.*—Previous investigators have demonstrated that normal platelets have antihemophilic[5] and proaccelerin-like[20] activity. Moreover, the platelets of patients with parahemophilia have been shown to be deficient in proaccelerin-like activity,[3] and those of patients with hemophilia, deficient in antihemophilic activity.[5] These observations were confirmed in the following experiments. In addition, the platelets of the patient with combined hemophilia and parahemophilia were deficient in both antihemophilic and proaccelerin-like activities.

The results of the thromboplastin generation test were compared, using reagents prepared from the blood of each of the patients; a patient with classic hemophilia and a normal individual. The thromboplastin generation test was abnormal in each of the 3 disorders when the incubation mixture consisted of calcium, normal serum, crude cephalin derived from rabbit brain, and aluminum hydroxide-adsorbed plasma obtained from the patient under study (Fig. 1). Conversely, the patients' sera behaved like normal serum when mixed with normal aluminum hydroxide-adsorbed plasma, crude cephalin, and calcium. In this system, then, the defect measured by the thromboplastin generation test was localized to the aluminum hydroxide-adsorbed plasma.

When normal platelets were substituted in the incubation mixture for crude cephalin, the abnormality measured in the thromboplastin generation test was less marked in each case (Figs. 2 to 5). In one experiment (Fig. 2) the thromboplastin generation test was normal in a mixture containing normal



Figs. 1 to 3.—In this and the succeeding figures, the results of the thromboplastin generation test are plotted in the usual manner. An "incubation mixture" of dilute aluminum hydroxide-adsorbed citrated plasma ("al. plasma"), dilute serum, crude cephalin or platelets, and calcium chloride was incubated at 37° C. At the intervals indicated on the abscissa, an aliquot of the incubation mixture was added to a "substrate" of citrated plasma, and this second mixture was then immediately recalcified. The clotting time of the substrate mixture is indicated on the ordinate. Each substrate tube was observed for 45 seconds, after which the tube was discarded even though its contents were unclotted.

Lower curve (Fig. 1). The thromboplastin generation test in parahemophilia and combined hemophilia and parahemophilia. The incubation mixture contained normal serum and crude cephalin in addition to the aluminum hydroxide-adsorbed plasma under test. The substrate was normal plasma. "Thromboplastin generation" in the presence of antihemophilic-deficient plasma, not shown here, fell between that of proaccelerin-deficient plasma and that of plasma deficient in both these factors.

Middle curve (Fig. 2). "Thromboplastin generation" in the presence of normal platelets. Normal platelets and serum were mixed with the aluminum hydroxide-adsorbed plasmas under test. The substrate was normal plasma. "Thromboplastin generation" in the presence of hemophilic aluminum hydroxide-adsorbed plasma was similar to "generation" with proaccelerin and antihemophilic-deficient plasma.

Upper curve (Fig. 3). A comparison of normal platelets and combined parahemophilic and hemophilic platelets. The incubation mixture contained aluminum hydroxide-adsorbed plasma from the patient with combined deficiencies of proaccelerin and antihemophilic factor, normal serum, and the platelets to be tested. The substrate was normal plasma. An all-normal system clotted in 10 seconds after 3 minutes' incubation.

platelets, normal serum, and aluminum hydroxide-adsorbed plasma obtained from the patient with parahemophilia. These experiments confirm the view that normal platelets exhibit proaccelerin-like activity.

On the other hand, "thromboplastin generation" was uniformly abnormal when the incubation mixture contained the patient's platelets along with his

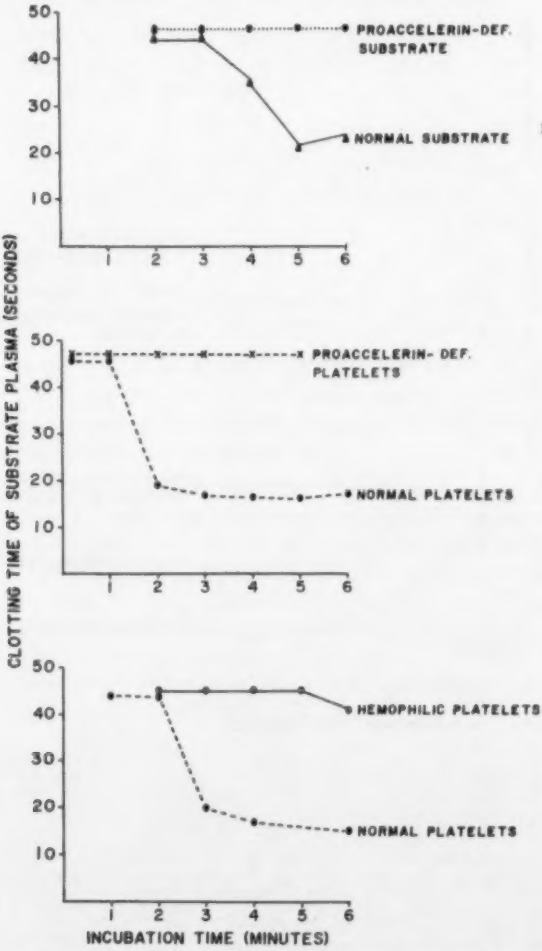


Fig. 6.

Fig. 5.

Fig. 4.

Figs. 4 to 6.—*Lower curve* (Fig. 4). A comparison of normal and hemophilic platelets. The incubation mixture contained hemophilic aluminum hydroxide-adsorbed plasma, normal serum, and the platelets to be tested. The substrate was normal plasma. An all-normal system clotted in 10 seconds after 3 minutes' incubation.

Middle curve (Fig. 5). A comparison of normal and parahemophilic platelets. The incubation mixture contained parahemophilic aluminum hydroxide-adsorbed plasma, normal serum, and the platelets to be tested. The substrate was parahemophilic plasma. An all-normal system clotted in 10 seconds after 3 minutes' incubation.

Upper curve (Fig. 6). A comparison of normal and parahemophilic substrates in the study of parahemophilia. The incubation mixture consisted of proaccelerin-deficient aluminum hydroxide-adsorbed plasma, and normal serum and platelets. An all-normal system gave a clotting time of 15 seconds after 6 minutes' incubation.

aluminum hydroxide-adsorbed plasma and normal serum. Thus, compared with normal platelets, hemophilic platelets were deficient in antihemophilic activity (Fig. 4), parahemophilic platelets were deficient in proaccelerin-like activity (Fig. 5), and platelets from the patient with the combined deficiency were less effective than normal platelets (Fig. 3), although by no means inert.

In the experiments illustrated in Figs. 1 through 4, the active principle or principles generated in the incubation mixture were tested upon a substrate of normal citrated plasma. In parahemophilia, the defect measured by the thromboplastin generation test was greatly exaggerated by the use of parahemophilic plasma as the substrate (Fig. 6). A substrate of parahemophilic plasma also exaggerated the defect when aluminum hydroxide-adsorbed plasma from the patient with the combined defect was tested in the incubation mixture.

The events which transpire in the incubation mixture, during the first stage of the thromboplastin test, are not clear. In one series of experiments, tissue thromboplastin prepared from rabbit brain was substituted for platelets or crude cephalin in the incubation mixture (Fig. 7). As in the case of the usual thromboplastin generation test, successive aliquots of the incubation mixture had progressively more acceleratory activity when added to a substrate of normal citrated plasma. However, the events moved more rapidly; in the presence of normal aluminum hydroxide-adsorbed plasma and normal serum, maximal activity was observed within a minute. No significant difference was observed in the behavior of the incubation mixture when hemophilic aluminum hydroxide-adsorbed plasma was substituted for normal adsorbed plasma. However, when parahemophilic adsorbed plasma from Case 1 or 2 was used, maximal activity evolved more slowly and the clotting time of the substrate was considerably longer than in the normal system. When normal serum was omitted from the generation mixture, maximal activity was not reached for 4 or 5 minutes in the presence of either normal or parahemophilic adsorbed plasma. When thromboplastin and calcium were incubated alone, successive aliquots were equally effective upon the substrate, as if no significant alteration occured in the incubation mixture in the absence of serum and adsorbed plasma.

The same results were obtained when human brain thromboplastin or Russell's viper venom was substituted for crude cephalin in testing parahemophilic plasma.

4. *The Effect of Normal Plasma Upon the Properties of Platelets Deficient in Antihemophilic and Proaccelerin-like Activity.*—Hjort, Rapaport, and Owren[4] demonstrated that the proaccelerin deficiency of parahemophilic platelets could be corrected by incubating the abnormal platelets with normal platelet-deficient plasma. These results were confirmed in studies of the platelets obtained from Patient 1, using the thromboplastin generation test. Similarly, it was possible to correct the defect in the platelets obtained from a patient with hemophilia and from Patient 2, who had the combined deficiency of antihemophilic factor and proaccelerin (Figs. 8 and 9). These observations

suggest that the antihemophilic activity of normal platelets, like their proaccelerin-like activity, is the result of adsorption of antihemophilic factor from the plasma.

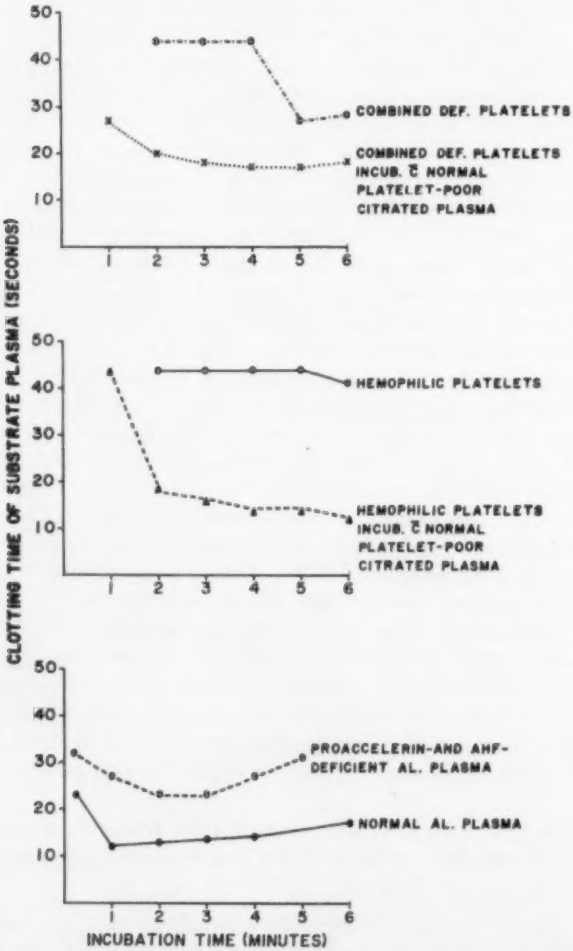

CLOTTING TIME OF SUBSTRATE PLASMA (SECONDS)
INCUBATION TIME (MINUTES)
COMBINED DEF. PLATELETS
COMBINED DEF. PLATELETS INCUB. C̄ NORMAL PLATELET-POOR CITRATED PLASMA
HEMOPHILIC PLATELETS
HEMOPHILIC PLATELETS INCUB. C̄ NORMAL PLATELET-POOR CITRATED PLASMA
PROACCELERIN-AND AHF-DEFICIENT AL. PLASMA
NORMAL AL. PLASMA

Fig. 9.

Fig. 8.

Fig. 7.

Figs. 7 to 9.—*Lower curve* (Fig. 7), The influence of tissue thromboplastin on the thromboplastin generation test. The incubation mixture contained normal adsorbed plasma or plasma from the patient with combined hemophilia and parahemophilia, normal serum, and rabbit brain thromboplastin. The substrate was normal plasma. An incubation mixture containing hemophilic instead of normal adsorbed plasma gave the same curve as normal plasma. Substitution of parahemophilic plasma gave a curve similar to that of the plasma deficient in both antihemophilic factor and proaccelerin.

Middle curve (Fig. 8), The effect of treating hemophilic platelets with normal plasma. The incubation mixture contained hemophilic aluminum hydroxide-adsorbed plasma, normal serum, and either hemophilic platelets or the same platelets treated with normal plasma. The substrate was normal plasma.

Upper curve (Fig. 9), the effect of treating platelets from the patient with combined deficiencies of proaccelerin and antihemophilic factor with normal plasma. The incubation mixture contained the adsorbed plasma from the patient with the combined deficiency, normal serum, and the platelets under test. The substrate was parahemophilic plasma but the same result was obtained with normal plasma.

DISCUSSION

The data which have been presented provide information concerning the relationship between hemophilia and parahemophilia, the nature of the platelets in these disorders and the interpretation of the thromboplastin generation test.

Parahemophilia, a congenital deficiency of proaccelerin, has been observed in about 20 families.[21, 22] Parahemophilia occurs in both sexes. Its mode of inheritance is not clear. In some instances, as in Case 1 of the present study, the parents or children of an affected individual have had partial deficiencies of proaccelerin. In others, as in Owren's original patient,[23] a deficiency of proaccelerin was not detected in any relatives. In contrast, classic hemophilia is virtually limited to the male and behaves as if it were transmitted as a sex-linked recessive trait.

In general, the bleeding tendency in parahemophilia parallels the degree of the defect measured in the laboratory. In the milder cases, the patients have had spontaneous epistaxes, easy bruisability, menorrhagia, and, at times, excessive bleeding after dental extractions or surgical procedures. In more severe cases, the patients have also had large hematomas, spontaneous gingival bleeding, and bleeding into the gastrointestinal tract or central nervous system. To our knowledge, hemarthroses have not been described. Except for the absence of hemorrhage into joints, these symptoms do not differ qualitatively from those of classic hemophilia.

Hemophilia and parahemophilia are so uncommon that one would expect their simultaneous occurrence to be exceedingly rare. Nevertheless, Case 2 of the present report represents the fourth recognized instance of this combination. Oeri and his associates[1] described two brothers with relatively mild hemorrhagic phenomena in whom the two abnormalities coexisted. The same coincidence was noted by Iversen and Bastrup-Madsen[2] in a mentally retarded boy with severe bleeding, hemarthroses, and priapism. The coexistence of deficiencies of antihemophilic factor and proaccelerin raises the question as to whether these two defects may result from a single abnormality. All 4 cases have been in males. The 3 previously reported instances resulted from consanguineous unions[1, 2] and the parents of our patient came from the same small village. A sister of one patient had hypoproaccelerinemia; his parents and two other siblings were normal.[2] In our case, the patient's mother had a partial deficiency of proaccelerin, but his only other available relative, a maternal half-brother, was normal. One explanation, suggested by Oeri and his associates,[1] is that a single gene is responsible for the synthesis of a precursor common to both antihemophilic factor and proaccelerin. An abnormal allele of this gene may result in impaired synthesis of this precursor. The evidence suggests that this allele is recessive compared with the normal allele. When an individual inherits a normal allele from one parent and the abnormal allele from the other, he synthesizes normal amounts of antihemophilic factor and either normal or nearly

normal amounts of proaccelerin. When the abnormal allele is inherited from both parents, the patient is unable to synthesize appreciable amounts of either protein. This hypothesis assumes that a second gene is required to convert the common precursor to proaccelerin and a third for conversion to antihemophilic factor. Were this the case, one should find instances of severe parahemophilia in which the concentration of antihemophilic factor is normal. In fact, antihemophilic activity has been normal in the few instances in which it has been tested,[3, 22, 24] and was normal in Case 1 of the present report. Conversely, the proaccelerin-like activity of the plasma of patients with classic hemophilia has been normal in our experience.

Lewis[3] and Mann[5] observed that the platelets of patients with parahemophilia or hemophilia were deficient in proaccelerin-like activity or antihemophilic activity, respectively. These observations have been confirmed. In addition, platelets prepared from the blood of the patient with combined hemophilia and parahemophilia were deficient in both antihemophilic and proaccelerin-like activities. Hjort and his associates[4] were able to correct the defect in parahemophilic platelets by exposing them to normal plasma. This observation, too, has been confirmed. In addition, the abnormalities of hemophilic platelets and of platelets prepared from the blood of the patient with the combined defect were corrected by the same technique. These studies suggest that both the antihemophilic and proaccelerin-like activities of normal platelets result from the adsorption of these factors from the plasma.

The thromboplastin generation test of Biggs and Douglas[15] was most useful in the study of patients described in the present report. The incubation mixture in this test contains all the known clotting factors, although it is relatively deficient in prothrombin. The active principle evolved in the incubation mixture is tested for "thromboplastic" activity upon a substrate of normal plasma. It was observed that the sensitivity of this test was much increased when the patient's own platelets were used instead of normal platelets in the incubation mixture. Furthermore, the thromboplastin generation test was much more sensitive in parahemophilia when the activity evolved in the incubation mixture was tested upon a substrate of parahemophilic plasma rather than normal plasma. In contrast, the abnormality of hemophilic plasma was independent of the nature of the substrate.

The nature of the active principle evolved in the thromboplastin generation test is not clear. The defect in parahemophilic plasma could still be detected when tissue thromboplastin was substituted for platelets in the first stage of this test. On the other hand, the abnormality in hemophilic plasma was corrected when tissue thromboplastin was substituted for the platelets in the incubation mixture. These observations suggest that the thromboplastin generation test does not, in fact, measure solely the evolution of a substance comparable in its activity to tissue thromboplastin. An alternate hypothesis is that a series of reactions occur, such as have been postulated by Macfarlane[25] and others. The first step in the reactions measured by this test involves the successive interactions of antihemophilic factor, Christmas factor, Hageman factor,

platelets (or "cephalin"), and calcium ions. A principle is thus evolved which is similar in behavior to tissue thromboplastin. This principle then reacts with proaccelerin to form the reagent measured in the second stage of the test. These observations do not elucidate the site of activity of pro-SPCA and Stuart factor, nor do they shed light on the nature of the active principle evolved in the first step of the thromboplastin generation test.

Finally, these studies emphasize the importance of looking for multiple defects in the plasma of patients with hemorrhagic disorders. The demonstration of these multiple defects may help define the steps which occur in the synthesis of the various factors involved in the coagulation of the blood.

SUMMARY

Cases of parahemophilia and of coexistent hemophilia and parahemophilia are described. The coexistence of these two defects suggests a common step in the syntheses of antihemophilic factor and proaccelerin.

That the hemophilic platelets are deficient in antihemophilic activity and parahemophilic platelets in proaccelerin-like activity has been confirmed. These defects were correctible by incubating the abnormal platelets with normal plasma.

The defect measured in the thromboplastin generation test was exaggerated in parahemophilia by the use of parahemophilic platelets and a substrate of parahemophilic plasma. The significance of these observations in relationship to the mechanisms measured by this test is discussed.

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STERILE-VOIDED URINE CULTURE

An Evaluation in 100 Consecutive Hospitalized Women

A. Donald Merritt, M.D., and Jay P. Sanford, M.D.
Durham, N. C.

URINARY tract infections remain among the most common diseases in which antibacterial therapy is useful.[1, 2] The more significant form of infection, pyelonephritis, is recognized clinically in only one-fifth of the 12 to 20 per cent incidence found at autopsy.[1, 3, 4] It is well known that serious infection may be entirely asymptomatic or be accompanied by supposedly inconsequential discomfort or pyuria.[4-6] Excluding those with active infection, Kass has recently shown that asymptomatic infections can be detected in at least 6 per cent of the female and 4 per cent of the male patients entering an outpatient department.[6] In certain groups of patients, such as those with diabetes mellitus, pregnancy, or cystocele, the occurrence of significant degrees of inapparent bacteriuria increases to almost one-quarter of those studied. It has previously been concluded that bacteriologic study alone can detect these inapparent infections.[2, 6, 9]

Bladder catheterization is the commonly accepted technique for obtaining urine from women for either examination of the sediment or culture. It has been widely appreciated that catheterization per se includes the hazard of introducing organisms into the bladder which are themselves capable of producing urinary tract infections.[2, 5-7] The incidence of occurrence of such iatrogenic infection is not precisely known; however, its occurrence is probably 2 to 3 per cent in women following catheterization. The disadvantage of this risk must be weighed against the possibility of overlooking clinically inapparent infections.

It is no longer common practice to catheterize the male patient in order to obtain an adequate urine specimen for examination and culture. Usual practice includes cleansing the glans penis and voiding with the collection of a midstream specimen. Even with fastidious collection of urine by voiding or catheterization, contamination must be distinguished from true bacteriuria. The use of quantitative bacterial counts of the urine makes this distinction possible.[6, 8] A midstream voided urine combined with quantitative bacterial counting of the urine culture and adapted to female patients seemed to offer a safe method of evaluation and screening. This study evaluated the feasibility of using "sterile-voided" rather than catheterized urines for culture in women.

From the Department of Medicine, Duke University School of Medicine, Durham, N. C.
Received for publication April 8, 1958.

Present address: National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md.

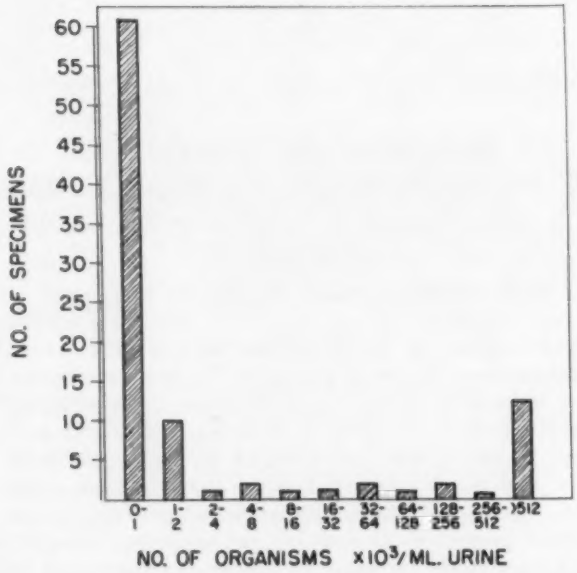

Fig. 1.—Frequency of varying degrees of bacteriuria in sterile-voided urines.

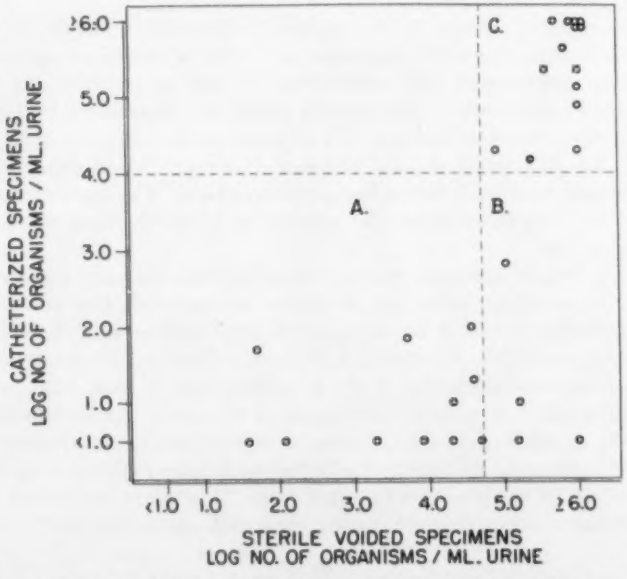

Fig. 2.—Comparison of degree of bacteriuria between sterile-voided and catheterized urines obtained from the same subject. The dividing lines are placed at 10,000 bacteria per milliliter for catheterized specimens and 50,000 bacteria per milliliter for sterile-voided specimens.

MATERIALS AND METHODS

Observations were made on 100 consecutive, ambulatory and nonambulatory, female admissions to a general public medical ward. One of us obtained a history from each patient which included: age, race, dysuria, frequency, nocturia, incontinence, urinary calculi, urinary infections, pelvic infections, pelvic operations, pregnancies, previous catheterizations, and diabetes mellitus occurring in the past or at the time of admission. Each patient was examined specifically for flank and suprapubic tenderness. Correlations of bacteriuria with the preliminary ward diagnoses, fever, leukocyte count, urinalysis, blood nonprotein nitrogen concentration, and phenolsulfonphthalein excretion were made.

"Sterile-voided" specimens were obtained from the patient on the day of admission by the ward personnel, both graduate and student nurses. The nurses received no special instructions, but were requested to prepare the patients for routine catheterization of the bladder, then, instead of inserting a catheter, to request the patient to void. A midstream specimen of urine was then collected. Preparation for routine catheterization consisted of placing the patient in the lithotomy position, with help if necessary, and cleansing the labiae and urethral orifice with Zephiran* sponges. The specimens were caught in standard 15 by 110 mm. sterile test tubes. The remainder of the urine was caught in a small basin commonly found on a bladder catheterization tray. The urine specimens were placed in an icebox (4° C.) within 5 minutes and quantitatively cultured within a 24-hour period. Specimens may be stored at 4° C. for as long as 48 hours without significant changes in the bacterial counts.[6]

Quantitative bacterial counts were carried out by making 3 successive tenfold dilutions of the urine in sterile 0.9 per cent saline solution. One milliliter aliquots of the 10^{-1} (1:10) and the 10^{-3} (1:1,000) dilutions were then pipetted into sterile Petri dishes. Approximately 9 ml. of melted, cooled, tryptic digest agar was added and mixed by swirling. The contents of the pour plates were allowed to cool and solidify, then inverted, and incubated at 37° C. for 24 to 36 hours. The bacterial colonies were then counted under magnification in a Quebec colony counter.

After removal of an aliquot of urine for quantitative bacterial count, the remainder of the sterile-voided specimen was sent to the bacteriologic laboratory for culture. Standard biochemical fermentation reactions and cultural characteristics were used for the identification of the bacterial strains isolated.

Urinalyses were performed by the ward medical staff and students on either sterile-voided specimens or routine specimens collected separately. When specimens other than those collected by the sterile-voided technique were utilized, they were collected within several hours of the cultured specimen. Again, no extra emphasis was placed on these urinalyses, and they were performed as a portion of the regular admission evaluation.

In all patients in whom the sterile-voided specimens were found to contain more than 5,000 colonies per milliliter and in some patients with fewer organisms, where the ward staff felt the procedure was indicated, catheterized specimens of urine were obtained. Such catheterized specimens were obtained within 24 to 36 hours of the sterile-voided specimens and usually prior to antibacterial therapy of any type. Laboratory studies were carried out on these specimens as described above.

RESULTS

The frequency of varying degrees of bacteriuria as demonstrated in sterile-voided urine specimens obtained from these patients was plotted (Fig. 1), forming a parabolic curve. Urine obtained from 61 patients had less than 1,000 bacteria per milliliter while that from 17 patients contained greater than 100,000 bacteria per milliliter. An essentially negative urine culture obtained

*Zephiran chloride (benzalkonium chloride), Winthrop Laboratories, New York, N. Y.

by the voided technique was obviously as meaningful as an "essentially negative" culture obtained by catheterization. The 25 patients in whom the sterile-voided urine contained greater than 5,000 bacteria per milliliter and 4 patients with lesser degrees of bacteriuria were catheterized. The correlation between a single sterile-voided urine and a single catheterized urine in the same patient was good (Fig. 2). Greater than 10,000 bacteria per milliliter of urine was present in 14 patients by catheterization (section C, Fig. 2). Each of these patients had greater than 50,000 bacteria per milliliter of urine on quantitation of their sterile-voided specimens. An additional 4 patients had greater than 50,000 bacteria per milliliter in their sterile-voided specimens while the catheterized urines obtained in these patients had less than 1,000 bacteria per milliliter (section B, Fig. 2). None of the 11 patients with sterile-voided urines containing less than 50,000 and greater than 10,000 bacteria per milliliter had significant degrees (greater than 10,000 bacteria per milliliter) of bacteriuria demonstrated in catheterized specimens. Thus, approximately 50,000 bacteria per milliliter could be used as the zone of differentiating contamination from significant bacteriuria when using sterile-voided urines alone. With this criterion an occasional "false positive" culture was obtained; however, no "false negative" cultures were observed. The bacterial species isolated from the sterile-voided and catheterized cultures were the same in 13 of the 14 positive cultures (Table I). In only one of the 15 negative cultures in which bacterial growth occurred, either sterile-voided or catheterized, was the same bacterial species isolated. In general, *Micrococcus pyogenes* var. *albus* was the most common contaminant, while *Escherichia coli* was the most common organism isolated in significant numbers. Yet *Esch. coli* as well as Proteus species were isolated as contaminants.

Significant bacteriuria (greater than 10,000 bacteria per milliliter of urine obtained by catheterization) was found in 14 of these 100 unselected consecutive admissions. The diagnosis of some type of urinary tract infection was suspected by the ward staff in 8 of the 100 patients. Of these, 5 were subsequently found to have positive cultures and 3 had negative cultures. In the 14 patients with positive cultures, 5 (36 per cent) had mention of a diagnosis concerning the urinary tract and in 9 there was no suspicion of urinary tract infection.

The clinical and laboratory features observed in the group with positive cultures were compared with those in the group of patients having negative cultures to ascertain whether or not there were indications suggesting relatively asymptomatic bacteriuria (Table II). There were 65 Caucasian patients and 35 Negro patients with a respective positive culture incidence of 15.4 and 11.4 per cent. More patients over the age of 60 years had positive cultures than younger patients; this difference was not statistically significant. It was of interest that cultures obtained from younger patients, particularly unmarried patients, were more apt to have higher degrees of contamination than those obtained from older, married patients. Urinary tract symptoms of

dysuria, frequency, or nocturia were commonly elicited from patients with positive cultures. Yet, these symptoms were not present in all patients with positive cultures and were elicited from a considerable number of patients with negative cultures. This triad of symptoms was present simultaneously in only 4 of the 14 patients (29 per cent) with positive cultures (Table III).

TABLE I. COMPARISON OF FINDINGS BETWEEN STERILE-VOIDED AND CATHETERIZED URINES

STERILE-VOIDED		CATHETERIZED	
BACTERIA/ML. X 10^{-3}	BACTERIAL SPECIES	BACTERIA/ML. X 10^{-3}	BACTERIAL SPECIES
0.04	N. G.*	<0.01	N. G.
0.05	N. G.	0.04	*M. albus*
1.20	N. G.	<0.01	*M. albus*
1.83	*Esch. coli*	<0.01	N. G.
5.04	*M. albus*	0.07	N. G.
8.30	Proteus sp.	<0.01	N. G.
21.0	*M. albus*	0.01	N. G.
21.0	*M. albus*	<0.01	Proteus sp.
37.0	N. G.	0.10	*M. albus*
38.0	*M. albus*	0.02	N. G.
50.0	*Esch. coli*	<0.01	N. G.
80.0	*Esch. coli*	20.0	*Esch. coli*
103.0	N. G.	0.64	N. G.
152.0	*M. albus*	<0.01	N. G.
157.0	*M. albus*	0.01	N. G.
233.0	*Esch. coli*	15.0	*Esch. coli*
384.0	*Esch. coli*	239.0	*Esch. coli*
> 500.0	*Esch. coli*	>1,000	*Esch. coli*
660.0	*Esch. coli*	448.0	*Esch. coli*
>1,000	*Esch. coli*	1,600	*Esch. coli*
>1,000	*Esch. coli*	20.0	Proteus sp.
>1,000	*Esch. coli*	>1,000	*Esch. coli*
>1,000	*Esch. coli*	140.0	*Esch. coli*
>1,000	*Esch. coli*	81.0	*Esch. coli*
>1,000	*Esch. coli*	>1,000	*Esch. coli*
1,460	*Strep. faecalis*	>4,000	*Strep. faecalis*
3,200	*Ps. aeruginosa*	228.0	*Ps. aeruginosa* *Esch. coli*
>5,000	*M. aureus*	<0.01	*M. albus*
>1,000	*Esch. coli*	>1,000	*Esch. coli*

*No growth.

A history of previous dysuria, frequency, or nocturia was obtained as often from patients with negative as with positive cultures. Likewise, the incidence of previous renal calculi, renal infections, pelvic infections, pelvic operations (usually an abdominal hysterectomy), and pregnancies was esssentially the same in each group. However, there were significantly more patients with diabetes mellitus in the group with positive cultures than in the group with negative cultures (Table IV). Catheterization in the past history was obtained with about equal frequency in both groups. No attempt was made to distinguish between a single catheterization and the use of an indwelling catheter in these patients. Urinalyses in each group were quite similar. There was no greater incidence of degree of pyuria using as criteria greater than 10, greater than 20, or clumps of leukocytes for comparison in those patients with positive cultures. An attempt was made to separate those with negative cultures from those with positive cultures by noting the combination of clinical observations,

TABLE II. COMPARISON OF CLINICAL AND LABORATORY OBSERVATIONS IN PATIENTS WITH NEGATIVE AND POSITIVE URINE CULTURES*

	SYMPTOMS (PER CENT OF PATIENTS)							
	PRESENT HISTORY				PAST HISTORY			
CULTURE	DYSURIA	FREQUENCY	NOCTURIA	INCONTINENCE	DYSURIA	FREQUENCY	NOCTURIA	INCONTINENCE
Negative	8.1	9.3	32.6	25.6	44.2	25.6	26.8	25.6
Positive	35.7	42.8	71.4	28.5	57.1	42.8	57.1	21.4
"p"	<0.02	<0.01	<0.02	—	—	—	—	—

	PAST HISTORY (PER CENT OF PATIENTS)						
CULTURE	CATHETERIZED	CALCULI	RENAL INFECTION	PELVIC INFECTION	PELVIC OPERATIONS	PREGNANCY	DIABETES MELLITUS
Negative	60.5	8.1	50.0	14.0	38.4	78.0	10.5
Positive	78.5	21.4	57.1	14.3	57.1	85.8	42.8
"p"	<0.5	<0.5	—	—	—	—	<0.01

	AGE (PER CENT OF PATIENTS)				LABORATORY OBSERVATIONS (PER CENT OF PATIENTS)					
						PYURIA			NPN	PSP
CULTURE	<20	21-40	41-60	>60	PROTEINURIA	0	<5	>5	>40 MG. %	<50 % IN 2 HRS.
Negative	9.3	38.4	43.0	9.3	12.8	18.6	60.5	20.9	18.7	23.0
Positive	0	28.5	42.8	28.5	28.5	7.1	64.2	28.5	18.2	10.0
"p"	—	—	—	<0.1	—	—	—	—	—	—

*Positive urine culture = >10,000 bacteria per milligram in catheterized specimens (14 per cent). Negative urine culture = <10,000 bacteria per milligram (86 per cent).

TABLE III. COMPARISON OF URINARY TRACT SYMPTOMS WITH QUANTITATIVE URINE CULTURES

	0 TO 1,000 BACTERIA/ML. (61)		NEGATIVE=<10,000 BACTERIA/ML. (86)		POSITIVE=>10,000 BACTERIA/ML. (14)	
SYMPTOMS	NO.	PER CENT	NO.	PER CENT	NO.	PER CENT
Dysuria, frequency, nocturia	0	0	1	1.2	4	29
Frequency, nocturia	3	4.9	5	5.8	6	43
Dysuria, pyuria	5	8.2	6	7.0	4	29
Frequency, pyuria	6	9.8	8	9.3	5	36
Nocturia, pyuria	18	29.6	22	25.6	9	64
Dysuria, frequency, pyuria	0	0	1	1.2	3	21
Dysuria, nocturia, pyuria	2	3.3	4	4.8	3	21
Dysuria, frequency, nocturia, pyuria	0	0	1	1.2	3	21
Frequency, nocturia, pyuria	3	4.9	5	5.8	5	36
Pyuria	51	83.6	70	81.5	13	93

TABLE IV. COMPARISON OF URINARY TRACT SYMPTOMS WITH QUANTITATIVE URINE CULTURES IN DIABETES MELLITUS (15 PATIENTS)

SYMPTOMS	NEGATIVE=<10,000 BACTERIA/ML. (9)	POSITIVE=>10,000 BACTERIA/ML. (6)
Dysuria present	3	3
Frequency present	1	2
Nocturia present	4	5
Pyuria present (>5 W.B.C.)	1	1
Previous catheterization	6	6

such as dysuria, frequency, nocturia, and the presence of leukocytes in the urine on admission to the hospital (Table III). Only one of 86 patients had this combination in that group with negative cultures, yet only 3 of 14 patients with positive cultures had these findings. It was found that no historical or laboratory evidence allowed one to separate patients with positive cultures from those with negative cultures except by the quantitative estimation of bacteriuria.

One of the patients had been catheterized two days prior to transfer to the medical service. The original culture had no bacterial growth; however, after catheterization, she developed dysuria and nocturia. A sterile-voided and a repeat catheterized specimen then contained *Esch. coli* in respective quantities of 600,000 and 500,000 organisms per milliliter. This was the only evidence of infection following catheterization (one in 29 catheterizations) in this small series.

DISCUSSION

The importance of a safe method for the bacteriologic evaluation of minor symptoms which may be indicative of significant bacteriuria is well illustrated by this group of patients. In the hospital or clinic as a routine screening procedure, particularly in women, this method is adequate for the evaluation of the urinary tract and particularly bacteriuria. A screening procedure seems indicated by the findings of significant bacteriuria in the absence of symptoms or pyuria. The presence of symptoms and/or pyuria needs similar evaluation, since these findings were present in the absence of significant bacteriuria.

While the over-all incidence of what is considered significant bacteriuria closely approximates the frequency with which pyelonephritis is observed at autopsy, this does not establish a causal relationship. MacDonald and coworkers recently studied the relation of bacteriuria to pathologic lesions of the urinary tract in 100 unselected adult autopsies.[9] The finding of true bacteriuria (defined by them as greater than 100,000 organisms per milliliter of urine) correlates well with the presence of pyelonephritis, and they suggest that such a relationship may also obtain during life. Again, they observed that pyuria, azotemia, and albuminuria were not reliable indices of the presence or absence of bacteriuria or pyelonephritis.

In this group of patients, as in patients who are catheterized, variable degrees of contamination during the collection of urine specimens is not uncommon. This fact in itself does not invalidate the method. A sterile or essentially negative (approximately 10,000 bacteria per milliliter or less) is highly significant. Also, a urine culture containing more than 50,000 to 100,000 bacteria per milliliter is strongly suggestive of bacteria within the urinary tract and not contamination. The method seems satisfactory, safe, and informative as a screening or diagnostic procedure. However, with preliminary evidence of a significant degree of bacteriuria, either careful repetition of a sterile-voided urine collection or catheterization is advisable before undertaking extensive urological or bacteriological diagnostic and therapeutic regimens.

SUMMARY

1. A relatively simple technique for obtaining urine cultures from female patients based on the use of sterile-voided urine collections and a quantitative culture technique is described and evaluated by comparison with catheterized urine collections.

2. Sterile-voided urine cultures containing greater than 50,000 bacteria per milliliter of urine correlate well with significant bacteriuria in catheterized specimens. A sterile-voided urine culture containing less than 10,000 bacteria per milliliter is as significant as a catheterized specimen containing similar numbers of bacteria.

3. Significant bacteriuria was detected in 14 of 100 consecutively evaluated hospital admissions. In only 5 of these patients was urinary tract infection suspected on the basis of clinical findings and urinalyses.

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LABORATORY METHODS

PROPOSAL FOR A CERTIFIED STANDARD FOR USE IN HEMOGLOBINOMETRY—SECOND AND FINAL REPORT

R. KEITH CANNAN, D.Sc.
WASHINGTON, D. C.

INTRODUCTION

IN 1953, the Hematology Study Section of the National Institutes of Health requested the Division of Medical Sciences of the National Academy of Sciences—National Research Council to explore the possibility of establishing a hemoglobin standard for general use throughout the country. In response to this request, the Division in 1954 organized an Ad Hoc Panel for the Establishment of a Hemoglobin Standard under its Subcommittee on Blood and Related Problems.

Dr. George Cartwright, School of Medicine of the University of Utah, accepted the Chairmanship of the Panel. Its members were Dr. David L. Drabkin, University of Pennsylvania Graduate School of Medicine; Lt. Col. William H. Crosby, Jr., Walter Reed Army Institute of Research; Dr. George Brecher, National Institutes of Health; Dr. Wallace Brode, National Bureau of Standards; Dr. Israel Davidsohn, Mt. Sinai Hospital, Chicago, representing both the College of American Pathologists and the American Society of Clinical Pathologists; and Dr. A. H. Neufeld, representing the National Research Council of Canada. In 1956, Mr. John Gould succeeded Dr. Brode, and in May, 1957, Dr. Bradley E. Copeland, New England Deaconess Hospital, Boston, and Dr. Donald Brown of the Hackensack General Hospital succeeded Dr. Davidsohn as representatives of the College of American Pathologists and the American Society of Clinical Pathologists, respectively. The Panel was also fortunate in having the cooperation of Dr. E. J. King of the Postgraduate Medical School of London, who has given his full support to this undertaking.

Most routine methods of clinical hemoglobinometry depend upon the photometric measurement of a blood sample after quantitative conversion of the hemoglobin which it contains into one or another of its derivatives. For the standardization of such a procedure there is needed a color standard which, when measured in the photometer and cuvette in routine use, will

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establish the relation between instrument reading and concentration of the particular hemoglobin derivative. This relation can then be used in converting instrument readings for unknown samples of blood to concentration of hemoglobin by the use of Beer's law or by construction of a calibration curve.

The problem, however, is complicated by the fact that several methods of analysis involving conversion to different forms of hemoglobin are in common use. Each of these methods should have its own standard. Since a multiplicity of standards is neither desirable nor practicable, the Panel decided, early in its deliberations, to select only one method of analysis for which a suitable direct standard would be developed. It should, however, be pointed out that this procedure does not preclude the use of the standard for the indirect calibration of another method. All that need be done is to construct a calibration curve based on the measurement of a small series of *normal* blood samples by the standardized method and also by the method which the analyst prefers to adopt for routine use.

In the opinion of the Panel, the most significant contribution which could be made to the refinement of clinical hemoglobinometry would be the widespread adoption of a single method of analysis. Failing this, the indirect calibration of other methods with the chosen method offers the only simple photometric means for the comparison of data. The procedure, however, is subject to error if the blood sample contains significant amounts of certain of the abnormal forms of hemoglobin. For example, methemoglobin and carbon monoxide-hemoglobin are quantitatively convertible to cyanmethemoglobin, but not to oxyhemoglobin.

The Panel reviewed the several photometric methods in current use and came to the conclusion that the procedure involving the measurement of hemoglobin as cyanmethemoglobin was the most promising. It offered the following advantages:

1. A simple and accurate procedure has been devised by Drabkin involving the addition of a single reagent to the sample of blood.[1, 2]

2. The method has been adopted by the U. S. Army after extensive field trials.[3]

3. All forms of hemoglobin likely to occur in circulating blood, with the exception of sulfhemoglobin, are determined by the method.

4. The color is suitable for measurement in filter-type photometers as well as in narrow band spectrophotometers because its absorption band in the region of 540 mμ is broad and relatively flat.

5. The U. S. Army has had extensive experience in the use of solutions of cyanmethemoglobin as direct standards and has found these to be satisfactory.[3] The U. S. Army standards have remained unchanged in optical density for extended periods when stored at refrigerator temperatures, provided bacterial contamination was avoided.

FIELD TRIAL

On the basis of this evidence, the Panel decided to develop a certified solution of cyanmethemoglobin as a standard, and to promote an extensive field trial of its suitability.* A preliminary report of its recommendations and plans appeared in a number of scientific and technical journals in 1955.[4-12] This report outlined recommendations of the Panel, described arrangements for the preparation and distribution of certified standard solutions of cyanmethemoglobin, and invited cooperation in an extensive field trial of the use of the standards and of the recommended method of analysis.

More than a thousand laboratories volunteered to cooperate in the trial sponsored by the National Research Council. Distribution of the standards was made with the assistance of the College of American Pathologists, the National Association of Clinical Laboratories, the Walter Reed Army Institute of Research, and the National Research Council of Canada. The laboratories not only received descriptions of the procedures for the use of the standards to calibrate photometers, but also directions for the routine determination of hemoglobin in the form of cyanmethemoglobin.

RESULTS

The results of the study were most gratifying. The need for, and the ready and grateful acceptance of, a simple method for the standardization of hemoglobinometers was apparent. The recommended method of analysis was likewise well received. At the onset of the field trial study only 7 per cent of the cooperating laboratories had been determining hemoglobin as cyanmethemoglobin. At the time of the last report, two-thirds of the cooperating laboratories were using this method.

In the first field trial, the standard solutions were prepared from crystalline hemoglobin by Dr. David Drabkin. Three solutions in carefully determined concentrations of approximately 60, 40, and 20 mg. of hemoglobin per 100 ml. were distributed. The optical densities of the final solutions were independently confirmed and a continuing control on stability was maintained in the laboratories of Dr. Brecher, Mr. Gould, Col. Crosby, Dr. King, and Dr. Neufeld. Agreement having been reached on the optical density values, the hemoglobin concentrations of the standards in milligrams per cent were computed from the optical densities, assuming that the extinction coefficient of cyanmethemoglobin per milligram-atom of iron (55.85 mg.) per liter is 11.5 and that the pigment contains 0.335 per cent of iron.

Two problems were encountered during the course of the field trial study. The first of these was the growth of certain microorganisms observed in some samples in spite of the presence of cyanide. This made it necessary to prepare and to maintain the solutions under sterile conditions. The second problem was a change, unpredictable in degree and not reported by all checking laboratories, of 2 to 6 per cent in the optical density 6 to 9 months after distribution of the standard. Samples from each lot were found to have undergone

*Proteins are not generally regarded as highly stable in dilute solution, even if sterile. In spite of the experience of the U. S. Army, the Panel hesitated to adopt a standard to which theoretical objection might be taken and explored other more stable materials. These included colored glasses and solutions of pigments so prepared as to approximate the absorption by cyanmethemoglobin of the spectral transmission of the filters, prisms or gratings employed in photometers and spectrophotometers. These alternatives were rejected because (a) no suitable mixture of pigments suggested itself, (b) it would be necessary to provide a series of glass standards to match the sizes and shapes of commonly used cuvettes, and (c) for some instruments the glasses would also have to match the lens effect of the round cuvettes and their contents.

varying degrees of change, mostly fading, which was compensated in some samples by a comparable increase in turbidity. The standards prepared and distributed by the U. S. Army in its earlier field trial had remained unchanged in optical density for 3 years. Inasmuch as these solutions had been prepared directly from whole blood or from washed red cells, it was suspected that the manipulation involved in the preparation of the crystalline hemoglobin for the National Research Council standards might have reduced the stability of the pigment. Therefore, a new standard was prepared from washed cells. The new standard was further modified by increasing the concentration of cyanide, since some previous preparations with such higher concentrations had shown greater stability and since the growth of most organisms would be limited by the higher concentration of cyanide.

Since the percentage change in optical density of the first standards was greater with increasing dilution of the hemoglobin pigment, and since cyanmethemoglobin solutions follow Beer's law, the second standard was distributed in only the most concentrated (60 mg. per cent of hemoglobin) of the 3 dilutions.

The second group of standards, modified as outlined above, was distributed in July and August of 1956. Their stability was determined in the laboratories of Dr. Drabkin, Dr. Brecher, Mr. Gould, and Col. Crosby. The stability was satisfactory for at least 9 months from the time of preparation, no change of more than 2 per cent in optical density being observed.

DISCUSSION

The members of the Panel have concluded that solutions of cyanmethemoglobin, when prepared, calibrated, and handled properly, are acceptable as standards for hemoglobinometry. They recognize that such standards are not ideal in all respects. However, until better standards can be developed, they are of the opinion that the availability of this reagent will greatly simplify the calibration of hemoglobinometers and will greatly increase the accuracy of hemoglobinometry over previously employed practices.

Finally, they encourage further independent investigation in the hope that an even better standard may be developed, particularly one with improved stability and more certain maintenance of sterility.

The National Research Council supplies of the standard cyanmethemoglobin solution are now exhausted and no further production is planned under the auspices of the Academy-Research Council. However, standards are now available from several commercial sources. The NAS-NRC has recommended the establishment of a program of certification of commercially produced cyanmethemoglobin standards to determine conformance with the specifications it has established. In response to the need for the establishment of such a program, as defined by the NAS-NRC, the College of American Pathologists has arranged for certification through the facilities of the laboratory of the American Medical Association in Chicago. On the basis of data obtained

through this laboratory, the College of American Pathologists will certify whether commercially produced standards which have been submitted comply with the specifications established by the NAS-NRC. *All users are urged to insist that the cyanmethemoglobin standards they purchase commercially carry the certification label of the College.*•

Detailed instructions for the preparation of the standards have been published by Crosby.[13] Producers of the standard or instrument manufacturers may obtain technical details on the adaptation to and use of the standard in the various hemoglobinometers by writing to the Division of Medical Sciences of the National Research Council.

RECOMMENDATIONS

The final recommendations of the National Research Council Ad Hoc Panel on the Establishment of a Hemoglobin Standard are as follows.

1. That cyanmethemoglobin be adopted as a standard in clinical hemoglobinometry.

2. That the standard be characterized spectrophotometrically on the basis that the extinction coefficient of one milligram-atom of iron (c = 1 mg. atom of iron per liter, d = 1 cm.) in the form of cyanmethemoglobin at a wave length of 540 mμ is 11.5.

3. That 0.338 per cent (w/w) be accepted as the iron content of hemoglobin (molecular weight of 16,520 per gram atom of iron) in accordance with the recent recommendation of the Protein Commission of the International Union of Pure and Applied Chemistry, and that a factor of 1,652 be used in calculating hemoglobin in milligrams per 100 ml. from millimoles per liter.

4. That the standard be distributed as a single concentration of not less than 55 mg. of cyanmethemoglobin per 100 ml.

5. That solutions be distributed in brown glass containers and in sterile condition.

6. For the present it is recommended that solutions should be used as standards for a period not to exceed 9 months from the time of preparation. This dating period is based upon the results of the National Research Council field trial. As experience accumulates with commercially prepared samples, an extension of the dating period may well be found to be justifiable.

7. That the standard be prepared from either crystalline hemoglobin or washed erythrocytes.

8. That commercial producers of the standards submit representative specimens from each lot to the College of American Pathologists, Prudential Plaza, Chicago 1, Illinois, for certification (a) that the concentration of cyanmethemoglobin is within ± 2 per cent of the value stated on the label; (b) that the solution is substantially optically clear; and (c) that it is microbiologically sterile.

•In Canada, discussions are now taking place in order to make one national laboratory responsible for the production and certification of the standard solutions.

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SPECTROPHOTOMETRIC DETERMINATION AND DISTRIBUTION OF CHLOROQUINE IN HUMAN TISSUES

RICHARD W. PROUTY, B.S.,* AND KEIJI KURODA, B.S.
UNITED STATES ARMY MEDICAL CENTER, JAPAN

JOSEPHSON and associates[1] reported a method for the estimation of many basic organic compounds in biologic materials that exhibit characteristic absorption maxima in the ultraviolet region. This method has generally wide applications to those compounds not readily amenable to analysis by other methods. Brodie and co-workers[2] developed a specific and sensitive means for estimation of antimalarials including chloroquine that can be converted to fluorophores when subjected to ultraviolet irradiation under suitable conditions. Berliner and associates[3] made studies on physiologic disposition of 4-aminoquinoline derivatives, in which they presented certain data on distribution of chloroquine in tissues and body fluids in rats. However, there is little information available on distribution of the base in human tissues. The present authors[4] have already demonstrated that chloroquine base can readily be detected in extracts by paper chromatography and that the amounts of metabolic products present in tissues are negligible compared with that of the parent drug, although Titus and co-workers[5] demonstrated the presence of considerable amounts of degradation products in the urine of subjects receiving the drug.

The present paper is concerned with ultraviolet spectrophotometric determination of chloroquine in tissues, and an attempt was made to establish distribution of the base among human organ tissues. The compound is isolated from the tissues by homogenizing with twice quantities of 0.1 hydrochloric acid, autoclaving the homogenate, and extracting with chloroform in alkalinized solution. The base in chloroform is finally extracted into 1 N hydrochloric acid. Spectrophotometric measurements of the final extracts are made using the Beckman Model DU spectrophotometer. The ultraviolet absorption spectrum of choroquine in 0.1 N hydrochloric acid is given in Fig. 1. The base possesses absorption maxima at the wave lengths 257, 330, and 342 mμ; measurements are most favorably made at 342 mμ, at which the optical density is the greatest and the absorption due to the base is least affected by interfering materials present in tissues.

MATERIALS AND METHOD

Reagents.—

1. Standard solution of chloroquine, 100 mg. base per liter. 100 mg. of the free base are dissolved on 1 liter of 0.1 N HCl.

From the Department of Chemistry, 406th Medical General Laboratory, APO 343, San Francisco, Calif.
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*First Lieutenant, Medical Service Corps, United States Army.

2. Potassium hydroxide, 16 N.
3. Chloroform.
4. Hydrochoric acid, 1 N and 0.1 N.
5. Sodium hydroxide, 0.1 N.

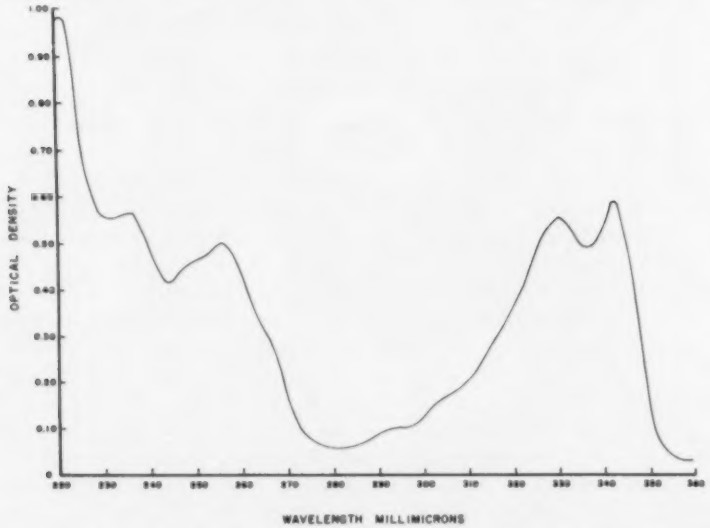

Fig. 1.—Absorption spectrum of chloroquine base (10 mg. per milliliter in 0.1 N. hydrochloric acid.

Procedure.—Fifty grams of organ tissues are homogenized with 100 ml. of 0.1 N hydrochloric acid in a Waring blendor, and 30 ml. of 40 per cent trichloroacetic acid is carefully added to the homogenate. After stirring the mixture well, 20 ml. of concentrated hydrochloric acid is added. Preferably the mixture is allowed to stand overnight to effect complete precipitation of proteins; it is then autoclaved under 15 pounds pressure for 30 minutes to liberate chloroquine bound to proteins and nucleic acids.[6] A large container should be used when autoclaving brain tissue in order to avoid overflow caused by gas evolution. The hydrolyzate is shaken once with 100 ml. of chloroform to remove fatty material. After thorough shaking the phases usually do not separate completely, even upon long standing. For complete separation the mixture is transferred to 250 ml. centrifuge bottles and centrifuged. This centrifugation will compress the precipitated proteins into a mass in the bottom of the bottle thereby facilitating the separation of the liquid phases. If the proteinaceous material is not separated completely at this stage, separation of the liquid phases in alkalinized medium at the next step will become exceedingly difficult owing to strong emulsification. The chloroform phase and the mass are discarded.

The aqueous phase is then strongly alkaline with potassium hydroxide and shaken with two 100 ml. portions of chloroform. The combined chloroform extract is washed with 50 ml. of 0.1 N sodium hydroxide and finally the chloroform phase is shaken with 25 ml. of 1 N hydrochloric acid.

The final acid extract shows the characteristic absorption spectrum of chloroquine when examined spectrophotometrically if the specimen contains more than 1 mg. of the base per

gram of tissue. A Beckman Model DK-2 recording spectrophotometer is useful for this purpose. The optical density of the extract is then measured, or dilutions of the same, using a Beckman Model DU spectrophotometer at 342 mμ.

Working standards of the drug are prepared by dilution of the standard stock solution with 0.1 N hydrochloric acid. The optical density follows Beer's law within the concentration range of 1 to 20 μg (Fig. 2).

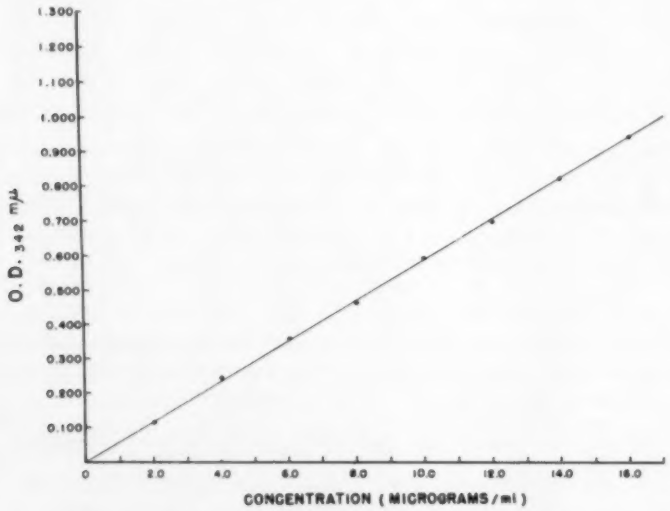

Fig. 2.—Relation of optical density to chloroquine base.

RESULTS

This laboratory routinely receives tissue specimens for toxicologic evaluation of deaths that occur among military personnel throughout Japan and in Korea. Many specimens from Korea have been found to contain chloroquine which was detected by paper chromatography.[4] Although it was known that the Security Forces in Korea were on antimalarial prophylaxis and that chloroquine was the agent most predominantly used,* it was indicated that quantitative studies be made because the circumstances surrounding the death of 2 of the 10 reported cases suggested suicide attempts with chloroquine. Table I lists the results of quantitation of the various submitted tissues by the above-mentioned procedure.

DISCUSSION

It is apparent from Table I that the highest concentration of chloroquine is found in the liver, followed by spleen, heart, and kidney. As expected, brain tissue disclosed the lowest level. In the two cases D and H, unusually high concentrations of the base were found. In most of the cases analyzed the liver

*One 500 mg. tablet of chloroquine diphosphate was used weekly from mid-April to mid-October yearly.

level was approximately 8 to 10 times the brain level and in case H the liver was found to contain 0.1 per cent by weight of chloroquine base as shown in Table I.

TABLE I. DISTRIBUTION OF CHLOROQUINE IN HUMAN TISSUES (MICROGRAMS PER GRAM)

TISSUE	A	B	C	D*	E	F	G	H*	I	J
Liver	58	57	6.2	–	24.0	13.0	5.5	900	4.3	29.0
Spleen	–	25	–	–	–	–	–	–	–	–
Heart	12	20	5.1	84	–	4.1	4.3	–	–	–
Kidney	–	–	0.6	–	–	3.2	3.4	470	2.0	5.8
Brain	7.3	6.9	0.5	10	3.0	1.0	0.7	11	1.7	2.0

*Reported suicide from ingestion of undetermined amount of chloroquine phosphate.

Although the amount of data available in this study is limited owing to the small number of cases studied and further investigations are necessary to establish normal distribution of the drug in human tissues conclusively, the scheme of analysis has proved to be most useful in estimation of the base isolated from extracts of human tissues.

SUMMARY

A rapid, sensitive procedure is presented for the estimation of chloroquine in biologic tissues. The distribution of the base in the various organs collected from 10 cases is discussed.

We would like to express our appreciation for the technical assistance rendered by Mr. Yuzuru Kato.

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A STUDY OF LIPIDS IN THE CEREBROSPINAL FLUID

II. The Determination of Total Lipids

Wallace W. Tourtellotte, Ph.D., M.D., Arthur J. Vander, A.B.,*
Beverley A. Skrentny, B.S., and Russell N. DeJong, M.D.
Ann Arbor, Mich.

In A recent review[1] we pointed out the necessity for a quantitative microchemical technique to determine total lipids in the cerebrospinal fluid (C.S.F.). The method described in this study permits the analysis of 250 μl of normal C.S.F. or as little as 2 μg of "total lipids," based on cholesterol oxidation.

The basic procedure adopted stems from the findings of Bloor.[2] He demonstrated that the colorimetric absorption produced by the reducing action of lipids on a dichromate-sulfuric acid reagent could be measured in the presence of nonreduced dichromate. As the oxidation is essentially nonspecific, the C.S.F. extract must be as free of nonlipid reducing substances as possible. By using the lipid extraction procedure of Robins and associates,[3] and by reducing drastically the volumes used in the analysis, it has been possible to increase the sensitivity of existing procedures 500 to 1,000 times.

In this paper the method will be described. Also experiments to test the validity of the method will be presented. These experiments include the range of the procedure, the linearity of extraction of lipids from different amounts of C.S.F., the determination of the coefficient of variation, and the comparison of the present procedure on diluted serum and lyophylized C.S.F. with the Sperry-Brand procedure.[4] Furthermore, a calculation for the neutral fat concentration has been derived.

MATERIALS AND METHODS

Reagents.—

1. Five and 50 per cent trichloroacetic acid (TCA) is vacuum distilled and diluted with glass double-distilled water.[5]

2. Potassium acetate in absolute ethanol. Two-tenths N potassium acetate is dissolved in redistilled absolute ethanol.

3. Phosphoric acid (H_3PO_4), analytical grade (85 per cent), is diluted with glass double-distilled water to 3 N.

4. Potassium dichromate-sulfuric acid reagent. Two grams of analytical grade potassium dichromate is dissolved in concentrated sulfuric acid with constant heating and stirring. The solution is then brought to a final volume of 100 ml. with concentrated sulfuric acid.

From the Department of Neurology, University Hospital, and Neurology Research Laboratory, Kresge Medical Research Building, University of Michigan Medical School, Ann Arbor, Mich.

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*University of Michigan Medical School student research fellow supported by the National Science Foundation.

5. Cholesterol (Eastman Kodak Company) was recrystallized. Standard cholesterol solutions of 25, 50, and 100 mg. per cent are prepared in redistilled absolute ethanol.

Apparatus.—

1. Lang-Levy pipettes are used throughout.[6]

2. Reaction tubes: 12 by 75 mm. Pyrex culture tubes without rim (Kimble 45060) are pointed to facilitate removal of supernatant fluid. Also, 7 by 70 mm. bacteriologic tubes without rims (Aloe 78140B) are used, but are not pointed.

3. Rubber stoppers to cap the 12 by 75 mm. tubes must be cleaned thoroughly with soap and water, rinsed completely with distilled water, and soaked in hot alcohol at least 12 hours and then dried before use.

4. Mixer.[6]

5. Centrifuge evaporator.[7]

6. A microsteam bath (1 by 3 by 7 inches) was designed to hold 55 7 by 70 mm. tubes (5 rows of 11). The chamber was made reasonably airtight except for a steam inlet and outlet and undersized holes on the top in a ⅛ inch thick piece of rubber. The unused holes must be stoppered to maintain uniform temperatures of 98 to 99° C. The part of the tube not immersed in the bath functions as a reflux condenser.

7. A Beckman spectrophotometer was adapted to read volume of 50 μl.*

Analytical procedure.—The precipitation and washing steps are carried out in an ice water bath.† The remaining steps are performed at room temperature. Lang-Levy pipettes are used for all transfers, and all mixings (buzzings) are accomplished by means of an off-center rotating cone or "buzzer."[8] The tubes must not be buzzed so vigorously that protein precipitate is thrown up along the sides of the tubes out of reach of the extracting reagents. A centrifuge evaporator[7] is used to remove solvents. With this device 30 samples can be evaporated simultaneously under reduced pressure without solvent creeping.

Step 1. Precipitation and washing: Three milliliters of C.S.F. is pipetted slowly to a pointed tube 12 by 75 mm. which contains 750 μl of 50 per cent TCA (final concentration of TCA is approximately 10 per cent). The tube is buzzed, capped with parafilm, and allowed to stand in the ice water bath for 15 minutes. The tube is buzzed again and centrifuged for 20 minutes at 2,600 r.p.m. in a cold room (4° C.). It is important after all centrifugations that the precipitate not be disturbed. After centrifugation, the supernate is removed and discarded. The precipitate is washed 2 times with 750 μl of 5 per cent TCA. Each tube is capped with parafilm and centrifuged 20 minutes at 2,600 r.p.m. in the cold. Again the supernate is removed and discarded.

Step 2. Lipid extraction: One hundred microliters of 0.2 N potassium acetate in absolute ethanol is added to the protein precipitate. The tube is buzzed immediately and removed from the ice bath. It is allowed to stand 10 minutes at room temperature, buzzed again, capped with parafilm, and centrifuged at room temperature for 20 minutes at 2,600 r.p.m. The supernate is transferred to a 1 ml. volumetric flask. The precipitate in the original tube is extracted again with 300 μl of absolute ethanol. The tube is buzzed, capped with parafilm, and centrifuged. The supernate from this wash is transferred to the 1 ml. volumetric flask. A third extraction of the precipitated protein is carried out in the same manner except, after the addition of 300 μl of ethanol, the tube is heated in a 60° C. water bath after capping with a defatted rubber stopper on parafilm. After centrifugation, the supernate is transferred again to the 1 ml. volumetric flask. The collected ethanol extracts are brought to a volume of 1 ml. with absolute ethanol.‡ A 250 μl aliquot is transferred to a 7 by 70 mm. tube. The tube is

*All equipment necessary to adapt the Beckman spectrophotometer to microanalysis was obtained from the Pyrocell Manufacturing Company, 207 East 84th Street, New York 28, N. Y.

†The cold condition decreases the possibility that some phosphate or organic base will be split from the lipids and therefore lost in the washing procedure.

‡Ample lipids remain in the volumetric flask after removal of the total lipid aliquot for simultaneous determination of total phospholipids[8] and free and total cholesterol.[9]

placed in the centrifuge evaporator and completely dried (60 to 90 minutes). The evaporation of the ethanol can be hastened by placing a 250 watt infrared lamp approximately 2 feet from the side of the evaporator.

Step 3. Removal of acetate: One hundred microliters of 3 N phosphoric acid is added to the dried contents of the tube and buzzed. The tube is placed in the centrifuge evaporator with the infrared lamp about 1 foot from the side for 90 to 120 minutes.

Step 4. Measurement of dichromate reduction: Forty microliters of the dichromate reagent is transferred to the tube. The tube is buzzed to cover all the residue. Each tube is covered tightly with aluminum foil and placed in a microsteam bath for 20 minutes. The reaction is stopped by placing the tube in an ice water bath for several minutes. The water condensed along the sides is brought down by a short centrifugation. Forty microliters of water is added and each tube is buzzed immediately. The reaction mixture is transferred to 1.5 mm. corex microabsorption cell (light path of 1 cm.) and the optical density of the sample is determined at 600 mμ in the Beckman spectrophotometer.

Step 5. Preparation of blanks and cholesterol standards: Two hundred microliters of 0.2 N potassium acetate in absolute ethanol is placed in a 2.0 ml. volumetric flask and brought to volume with absolute ethanol. Aliquots of 125 μl are placed in 12 separate 7 by 70 mm. tubes. The contents are completely dried in the centrifuge evaporator (60 to 90 minutes). Three tubes serve as the reagent blank. Thirty microliters of 25 mg. per cent of cholesterol is placed is 3 tubes, 30 μl of 50 mg. per cent of cholesterol in 3 other tubes, and 30 μl of 100 mg. per cent of cholesterol in the last 3 tubes. The ethanol in which the cholesterol is dissolved is then removed by means of the centrifuge evaporator. The blanks and standards are then carried through Steps 3 and 4 simultaneously with the tissue samples.

Comments on Analytical Procedure.—Step 1: An experiment was performed to determine whether the cold acid treatment carried out as described had removed significantly the acid-soluble substances from the protein precipitate. The supernate from the final TCA wash was dried and treated with the dichromate reagent. The optical density was determined at 600 mμ and found to be zero. This nonreduction of dichromate indicated that two TCA washes were sufficient to remove interfering acid-soluble contaminants.

TABLE I. EFFECT OF DIFFERENT AMOUNTS OF TRICHLOROACETIC ACID ON CHOLESTEROL OXIDATION BY DICHROMATE

TCA (μEq)	CHOLESTEROL μg				
	1.8	3.7	7.3	14.7	29.3
0	0.036	--	0.142	0.327	0.684
3.7	0.054	--	0.168	0.326	--
4.7	--	--	--	0.343	0.647
8.8	0.050	0.090	0.187	0.346	0.665
17.6	--	0.088	--	0.369	0.688

The indicated amounts of trichloroacetic acid and cholesterol were dried, reacted with dichromate reagent as previously described in Step 4, and the optical density was determined. Each recorded optical density is the average of triplicate determinations.

Because measured aliquots of the TCA supernates were not taken, varying amounts of TCA were left covering the protein precipitate. Therefore, the question arose whether TCA had any effect on dichromate reduction. Varying amounts of TCA were reacted with the dichromate reagent and the optical densities were measured. As much as 17.61 μEq of TCA gave no reduction of the dichromate reagent. Another experiment was designed to determine whether dichromate oxidation of different amounts of cholesterol was influenced by the presence of different amounts of TCA. The results are recorded in Table I. The data indicated that varying amounts of TCA did not produce any real effect on the dichromate-cholesterol reaction, since in a later section it will be shown that the coefficient of variation for the proposed procedure is between 5 and 10 per cent. It was observed that duplicate determinations were more reproducible if a small amount of TCA was present (3.7 μEq or

more). This was probably due to the fact that the presence of TCA decreased creeping and "bumping" of the ethanol extract during evaporation and, therefore, kept the lipid material at the bottom of the tube where it could more readily react with the dichromate reagent.

Step 2: The possibility of incomplete alcoholic extraction of lipids from the protein was tested. Chloroform and methanol (2:1) extraction of the protein mass after 3 alcoholic extractions as described above in the analytical procedure yielded 1.2 per cent more phosphorus (presumably phospholipid phosphorus). This experiment essentially confirmed the finding of more elaborate experimentation done on this point by Robins and associates.[3] Furthermore, an experiment was done in which a fourth hot alcoholic extraction (60° C.) was performed. This extract was dried, dichromate reagent was added, and the optical density was determined. No dichromate reduction was observed.

TABLE II. PER CENT OF PROTEIN SOLUBILIZED BY THE EXTRACTION PROCEDURE

C.S.F. PRECIPITATED PROTEIN (μg)	PROTEIN FOUND IN THE ETHANOL EXTRACT (μg)	PER CENT OF PRECIPITATED PROTEIN SOLUBILIZED
81.5	0.34	0.42
103.2	0.74	0.72
116.8	0.77	0.66
164.6	0.90	0.55
208.4	1.08	0.52
234.6	1.79	0.76
246.1	1.14	0.46
254.7	1.31	0.51
311.2	1.63	0.52
380.8	2.24	0.59
		Average 0.57 %

The lipids were extracted as mentioned in the analytical procedure. The combined ethanol extractions were dried and analyzed by means of Lowry and associates'[10] microprotein procedure. The protein in the original CSF sample was determined by means of a biuret reaction.[14]

When the lipids are extracted from the TCA protein precipitate, it is necessary to have an alkaline ethanol solution in order to minimize protein solubilization. A titration study of TCA with potassium acetate showed that a potassium acetate to trichloroacetic acid ratio of at least 2 is necessary to produce a slightly alkaline solution. An experiment was done using different potassium acetate to trichloroacetic acid ratios in the extraction steps. Protein determinations were then performed on the extracts.* The extract in which the ratio was 1 gave protein readings too high to be read by Lowry and associates' microprocedure.[10] The extract with a ratio of 2 showed slightly more protein than the extract with a ratio of 5. Robins and co-workers[3] used a ratio of 3.27. Though protein solubilization during extraction was greatly reduced by sufficient amounts of acetate, a certain amount of protein contamination seemed inevitable. In Table II is presented data to show that the amount of protein extracted with acetate in ethanol was almost directly proportional to the quantity of precipitated protein used. The average per cent protein solubilized was 0.57 per cent, with a range of 0.42 to 0.76.

In Fig. 1 a protein standard curve is shown.† The reaction appeared linear with a significant intercept. The equation of the line by the analysis of least squares[11] is $Y = -13 + 18.1\ X$, where Y is the reading (optical density × 1,000), where −13 is the intercept, where 18.1 is the slope, and X is the μg of protein in the sample. Because of the significant

*To determine the protein contamination of the ethanol extract, the extracts were analyzed by Lowry and associates'[10] microprotein procedure (sensitivity 0.3 μg of protein). The determination of protein by this method was complicated by the presence of potassium ions which combined with the sulfate ions of the Folin phenol reagent to give a precipitate. This precipitate was avoided by use of sodium acetate, rather than potassium salt to alkalinize the absolute ethanol solution. However, centrifugation before spectrophotometric analysis of the supernate was found to yield similar results.

†This equation of the line also applies to cerebrospinal fluid protein made free of lipid and acid-soluble material by the proposed procedure.

protein contamination of the ethanol extract and because proteins can reduce dichromate, it is necessary to correct the observed dichromate reduction of the ethanol extract for reduction, due to protein, to obtain the lipid concentration. These calculations will be shown in a subsequent section.

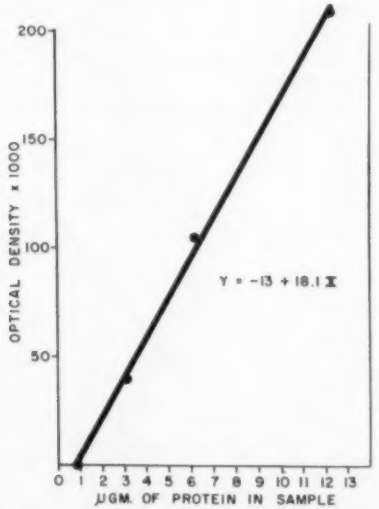

Fig. 1.—Effect of diluted Aloe lyophylized bovine serum protein on dichromate reduction. Various quantities of protein as indicated were dried in the centrifuge evaporator and reacted with the dichromate reagent as described in Step 4. The optical density is multiplied by 1,000 for the convenience of calculating the equation of the line.

Step 3: At the beginning of this step of the procedure the reagents have contributed 5 μEq of potassium acetate to each tube. The reduction of the contents of the blank tubes which contains only potassium acetate with the dichromate reagent gave high results (optical density of 0.210). The addition of silver ion and prolonged heating increased the blank reading further (optical density of 1.0). On the basis that this large amount of acetate was reducing the dichromate, we removed the acetate by vacuum distillation as acetic acid at 65° C. Four strong acids (phosphoric, nitric, sulfuric, and hydrochloric acid) were used separately to react with potassium acetate to give acetic acid and a salt of a strong acid. Three hundred microequivalents of phosphoric acid gave the lowest reagent blank (optical density of 0.110). This treatment had no effect on the cholesterol standard curve.

Standards.—Bragdon[12] has shown that oxidation of cholesterol, cholesterol stearate, palmitic acid, and triglycerides proceeded to the same degree in each case. The experiments of Bragdon were repeated, as directed in Step 4, after drying known amounts of recrystallized lipids. Based on the theoretical oxidation by dichromate* in which tristearin yields 3 acetates, cholesterol yields 4, and stearic acid yields 1, the data showed that tristearin, cholesterol, and stearic acid were oxidized 80, 77, and 75 per cent of the respective theoretical value. These results probably indicate that oxidation proceeded to the same degree

*Lemieux and Purves[13] have shown that oxidation by dichromate quantitatively recovered terminal methyl groups as acetic acid. This may seem to be contradictory to the previous paragraph (Step 3). However, we have found that the oxidation of acetate is dependent on the concentration because when various amounts of potassium acetate were reacted with the dichromate by the present procedure we found that less than 1 μEq of potasium acetate had no detectable effect on the optical density; whereas, 5 μEq of potassium acetate as mentioned previously had a very significant effect. Furthermore, when 29 μg (0.075 μM) of cholesterol (see Fig. 2) is reacted with dichromate to yield 4 acetate molecules and 78 per cent oxidation occurs, only 0.234 μEq of acetic acid is formed. This amount of acetic acid is less than can be detected with reliability.

in each case. No explanation could be found for the low per cent of oxidation in our experiments (average 77 per cent) compared to Bragdon[12] or Bloor,[2] 95 and 88 per cent of theoretical oxidation, respectively.

The color development of a cholesterol standard has been found to be quite constant over a period of 9 months and there is no necessity for standards once the spectrophotometer has been calibrated.

Fig. 2 illustrates a typical standard cholesterol curve with the present procedure. The reaction appears linear from 2 to 29 μg. The equation of the line by the analysis of least squares is $Y = -0.646 + 26.059\ X$. Inspection of the graph and the equation indicate that the intercept can be considered zero.

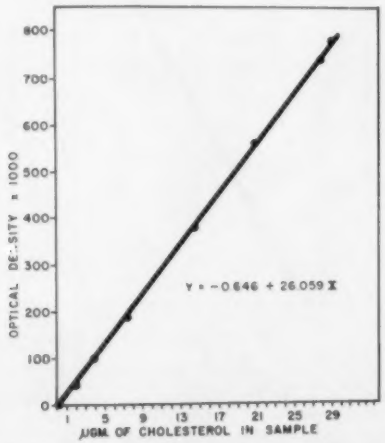

Fig. 2.—Cholesterol standard curve. Various quantities of cholesterol as indicated were dried in the centrifuge evaporator and then carried through Steps 3 and 4. The optical density is multiplied by 1,000 for the convenience of calculating the equation of the line.

Calculations of the Total Lipid Concentration Based on Cholesterol Oxidation.—The method of calculating the total lipid concentration based on cholesterol oxidation is presented below:

$$\frac{\text{O.D.} \times 100}{26 \times \text{aliquot volume}} - 0.004 \times \text{mg. per cent protein} = \text{mg. per cent lipid}$$

where O.D. is the optical density multiplied by 1,000; where 100 converts the results to milligrams per cent; where 26 is the slope of the cholesterol standard curve (Fig. 2); where aliquot volume represents the amount of spinal fluid used for the analysis; and where 0.004 × milligrams per cent protein represents the protein correction.*

RESULTS AND DISCUSSION

In Table III the reproducibility of the total lipid procedure is shown. Eleven or 12 determinations were made on two levels of two different specimens of C.S.F. (46 determinations). The average coefficient of variation was 7 per cent, with the coefficient being 5 per cent for the larger sample of C.S.F. and almost 10 per cent for the smaller.

*If the equations for the lines in Figs. 1 and 2 do not differ significantly from $Y = 18\ X$ and $Y = 26\ X$, respectively, then 1 mg. of protein in the spinal fluid is equivalent to 0.004 mg. of lipid ($0.006 \times \frac{18}{26}$, where 0.006 is the portion of the protein precipitate solubilized by the ethanol extract, Table II). The protein in the spinal fluid was determined by the method of Lowry and associates[10] or Tourtellotte and co-workers.[5, 11]

Experiments were also performed on different volumes of the same C.S.F. in order to determine whether the proposed extraction procedure would give the same concentrations. The same type of experiments were also performed on diluted serum. The results in Table IV show that the same concentration for a given C.S.F. or diluted serum is obtained over a wide range of volumes extracted (250 μl to 1,000 μl).

TABLE III. REPRODUCIBILITY EXPERIMENTS

DETERMINATION	C.S.F. 1		C.S.F. 2	
	500 μl	1,000 μl	500 μl	1,000 μl
1	0.927	0.949	0.869	0.861
2	1.007	0.962	0.878	0.843
3	0.936	0.954	0.799	0.979
4	1.104	0.980	0.834	0.874
5	1.024	1.007	0.957	0.878
6	0.936	1.015	0.905	0.891
7	0.909	0.949	1.036	0.799
8	0.901	1.051	1.001	0.843
9	0.998	0.993	0.808	0.856
10	0.954	1.033	0.773	0.865
11	1.024	0.914	0.738	0.786
12	1.166	--	0.948	--
Mean	0.991	0.982	0.879	0.861
Standard deviation*	±0.081	±0.041	±0.093	±0.050
Coefficient variation†	8.2%	4.2%	10.6%	5.9%

*Standard deviation = $\sqrt{\frac{\Sigma d^2}{n-1}}$.

†Coefficient of variation = $\frac{\text{standard deviation} \times 100}{\text{mean}}$.

All the values are expressed as milligrams of total lipid based on cholesterol oxidation per 100 ml. of C.S.F.

TABLE IV. EXTRACTION OF DIFFERENT VOLUMES OF C.S.F. AND DILUTED SERUM

		250 μl	500 μl	750 μl	1,000 μl
C.S.F.	1	--	0.88	--	0.86
	2	--	0.99	--	0.98
	3	1.91	1.73	--	--
	4	2.05	2.05	--	--
	5	2.39	2.20	--	--
	6	2.30	2.29	2.49	--
	7	2.80	2.46	2.45	--
Diluted serum	1	--	1.14	--	1.14
	2	--	1.75	--	1.37
	3	--	1.59	--	1.57
	4	--	2.08	--	2.17
	5	--	2.14	--	2.11
	6	--	2.31	--	2.21

The serum was diluted 1 to 200 with 0.85 per cent sodium chloride. Each figure is the average of triplicate determinations. The results are expressed in milligrams per 100 ml.

The final experiments performed were to test the quantification of the proposed procedure by comparison with the Sperry-Brand gravimetric procedure. The concentration of lipid in C.S.F. is several hundred times too low for analysis by the latter procedure. Therefore, 400 ml. of C.S.F. was pooled and, after aliquots were taken for lipid analysis by the proposed microprocedure, the fluid was

lyophylized. The dried material was extracted by the procedure of Brante[15] and the Sperry-Brand[4] procedure was carried out on the Brante extract. The proposed procedure yielded 88 per cent of the Sperry-Brand value. Furthermore, serum samples were analyzed for total lipids by the Sperry-Brand procedure. The serum was then diluted to a lipid concentration similar to that found in C.S.F. and the proposed procedure was carried out. The results in Table V indicate that the proposed procedure is quantitative.*

TABLE V. COMPARISON OF THE PROPOSED ANALYTICAL PROCEDURE WITH METHOD OF SPERRY AND BRAND[4]

SERUM	SPERRY METHOD	PRESENT METHOD
1	350	324
2	358	316
3	426	426
4	438	438
5	492	452

The Sperry-Brand gravimetric procedure was carried out on serum. The serum was diluted 200 times and the proposed procedure carried out. All values are expressed in milligrams per 100 ml. of the original serum.

In the oxidation-reduction reaction, each molecule of $K_2Cr_2O_7$ reduced produces 1 molecule of $K_2Cr_2(SO_4)_4$. A solution of potassium chrome alum equimolar to the dichromate reagent was prepared. A calibration curve was obtained by mixing varying proportions of an equimolar chrome alum and dichromate solutions. The broken line in Fig. 3 shows the curve obtained when 5 to 40 μl of the alum and 35 to 0 μl of the dichromate solutions are mixed. The optical density was determined as described in Step 4. The optical densities of the resulting mixtures are plotted against the amount of dichromate that was replaced (5 μl contains 100 μg of dichromate).

The theoretical dichromate-reducing power of various lipids is shown in Table VI. It was assumed that the oxidation goes to CO_2, H_2O, and acetate so that lecithin, cephalin, sphingomyelin, and the cerebrosides all yield 2 acetates, while tristearin, cholesterol, and cholesterol esters yield 3, 4, and 5 acetates, respectively.† In calculating the theoretical value for cholesterol esters, it does not matter which fatty acid is used since all fatty acids have almost identical reducing values. Standard cholesterol solutions containing 7 to 31 μg of cholesterol were reacted with the dichromate and the optical densities were determined as described in Step 4. The solid line in Fig. 3 shows the optical densities obtained from triplicate determinations plotted against the amount of $K_2Cr_2O_7$ that theoretically should have been reduced by the cholesterol. Tristearin and stearic acid (recrystallized from absolute ethanol) fell on the same line, indicating that oxidation proceeded to the same degree in each case.

*Furthermore, since the preparation of this manuscript, a more extended series has been done. The total lipid content of the serum from 21 fasting normal subjects was determined by both procedures (Proposed and Sperry-Brand). The means (mg. per cent) and s.e.m. were 875.6 ± 40.6 and 757.8 ± 43.3, respectively. The proposed procedure was 16 per cent higher than the Sperry-Brand method; this difference was statistically different (Student's "t" test on paired data, $p < 0.01$).

†These values differ from those of Bragdon because he assumed complete oxidation of lipids to CO_2 and H_2O.

The discrepancy between the lipid curves and that obtained with the alum solution shows that oxidation of the former was 78 per cent (75 to 80) of the theoretical value. It should be noted that this incomplete oxidation should have no effect on the total lipid calculations, since the lipids in the tissue samples and the standard used to obtain the calibration curve are oxidized under the same conditions.

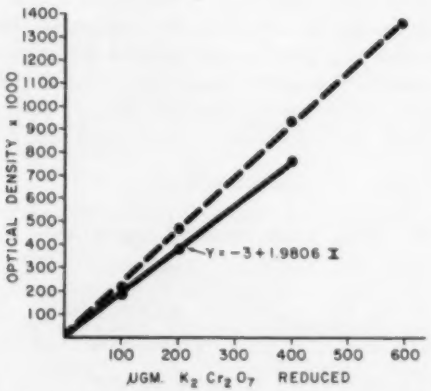

Fig. 3.—Calibration curves of lipid standards. The broken line represents the densities obtained when the quantities of dichromate in the abscissas are replaced by equimolar quantities of potassium chrome alum. The quantities of lipids (cholesterol, tristearin, and stearic acid) are expressed as micrograms of the $K_2Cr_2O_7$ they should have reduced (see text).

TABLE VI. THEORETICAL DICHROMATE-REDUCING POWER OF LIPIDS

LIPID	$K_2Cr_2O_7$ μg PER μg LIPID
Cholesterol	13.19
Cholesterol linoleic	14.96
Lecithin (1-α-phosphatidyl choline)	13.82
Plasmalogens	13.72
1-α phosphatidyl ethanolamine	13.75
1-α phosphatidyl serine	14.00
Cerebrosides (lignoceric acid glycosphingoside)	14.35
Sphingomeylin (lignoceric acid phosphosphingoside)	15.01
Palmitic acid	16.06
Linoleic acid	16.11
Stearic acid	16.55
Neutral fat (tristearin)	16.61

If the total cholesterol, total phospholipid and cerebroside content of the C.S.F. have been determined on aliquots of the same C.S.F. extract as the total lipid concentration, then the neutral fat content* of the C.S.F. can be calculated. The actual calculations are best illustrated by an example in which the various lipids are expressed in micrograms per 100 ml. of C.S.F.[16]: cephalins 91, lecithins 168, sphingomyelins 87, cerebrosides 59, esterified cholesterol 292, and free

*It is more correct to state that the difference between the total lipid and the sum of various components is the unaccounted for lipid fraction of which neutral fat may be a component.

cholesterol 109. The total lipid value based on cholesterol oxidation was 1,254 μg per 100 ml. The neutral fat content was calculated by subtracting from the total lipid value the sum of the various components (total cholesterol + total phospholipid + cerebroside). The neutral fat content was found to be 403 μg per 100 ml.

SUMMARY

An ultramicrototal lipid procedure is presented which is capable of determining 2 μg of lipids with a coefficient of variation of about 7 per cent. It is based on the oxidation of a suitable extract by a dichromate-sulfuric acid reagent and the colorimetric determination of the reduced chrome ion. Several tests for the validity of the proposed procedure on C.S.F. are presented. Calculations are presented for the derivation of neutral fats in C.S.F.

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A STUDY OF LIPIDS IN THE CEREBROSPINAL FLUID

III. The Determination of Total Phospholipids

Wallace W. Tourtellotte, Ph.D., M.D., Fannie M. Parker, A.B., and Russell N. DeJong, M.D.
Ann Arbor, Mich.

IT HAS been pointed out in a recent review[1] that phospholipids probably exist in normal cerebrospinal fluid (C.S.F.). Because of the very minute amounts of phospholipid phosphorus in normal C.S.F., i.e., 25 μg of phosphorus per 100 ml. of C.S.F., it is not surprising that some of the early investigators had great difficulty in finding it. Furthermore, in the C.S.F. the possibility of contamination of the phospholipid fraction with nonlipid phosphorus is very great because there is about 60 times more free phosphate (1.5 mg. of phosphorus per 100 ml.[2]) than phospholipid phosphorus. For this reason the C.S.F. lipid extract must be as free of nonlipid phosphorus as possible.

The basic procedure adopted stems from that of Lowry and associates.[3] The lipids are coprecipitated with the proteins, the protein precipitate is washed free of the acid-soluble phosphorus and the lipids are extracted with alcoholic acetate. An aliquot of the alcoholic extract is evaporated, wet ashed, and a sensitive phosphorus method ($E_{820} = 25{,}000$) is employed.

In this paper the method and experiments to test the validity of the method for determination of phospholipid in C.S.F. will be presented.

MATERIALS AND METHODS

Reagents.—

1. Trichloroacetic acid (TCA) is vacuum distilled and diluted to 5 and 50 per cent with water doubly distilled in glass apparatus.[4]

2. Potassium acetate in absolute ethanol; 0.2 N potassium acetate is dissolved in redistilled absolute ethanol.

3. Phosphate standards. Potassium phosphate, monobasic (KH_2PO_4) in 0.1 N sulfuric acid.

4. Ashing mixture: 0.8 N perchloric acid ($HClO_4$) in 10 N sulfuric acid.[3]

5. Color reagent for phosphate determination[3]; 0.25 per cent ammonium molybdate and 1 per cent ascorbic acid in 0.1 N sodium acetate, prepared just prior to use.

Apparatus.—

1. Lang-Levy pipettes are used throughout.[3]

2. Reaction tubes, 12 by 75 mm. Pyrex culture tubes without rim (Kimble 45060) are pointed to facilitate removal of supernatant fluid. Also 7 by 70 mm. bacteriologic tubes without rims* are used, but are not pointed.

From the Department of Neurology, University Hospital and Neurology Research Laboratory, Kresge Medical Research Building, University of Michigan, Ann Arbor, Mich.
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*No. 78140B, A. S. Aloe Co., St. Louis, Mo.

3. Centrifuge evaporator.[5]

4. Beckman spectrophotometer is adapted to read volumes of 50 μl.[6]

Analytical Procedure.—The precipitation and washing steps are carried out in an ice water bath. The remaining steps are performed at room temperature. Lang-Levy pipettes are used for all transfers, and all mixings are accomplished by means of an off-center rotating cone or "buzzer."[2] The tubes are not buzzed so vigorously that protein precipitate is thrown up along the sides of the tubes out of reach of the extracting reagents. A centrifuge evaporator[5] is used to remove solvents. With this device 30 samples can be evaporated simultaneously under reduced pressure without solvent creeping.

Step 1. Precipitation and washing: As previously described.[6]

Step 2. Lipid extraction: As previously described.[6]

Step 3. Wet ashing and the development of the phosphorus color: A 50 μl aliquot is removed from the volumetric flask and placed in a 7 by 70 mm. tube. This tube is placed in a centrifuge evaporator and dried (60 to 90 minutes). The evaporation of the ethanol can be hastened by placing a 250 watt infrared lamp approximately two feet from the side of the evaporator. Add 22.5 μl of 1:1 ashing mix and buz. The tube is placed in a 95° C. oven for 2 hours and subsequently in a 165° C. oven for 2 hours. If the tube is not clear, cool and add 5 μl of fuming nitric acid and return to the 165° C. oven for 30 minutes. Repeat the fuming nitric acid treatment until the tube is clear. Add to the cooled, cleared tube 117.4 μl of color developer and buz immediately after addition. The tube is capped with parafilm and placed in a 38° C. water bath for 2 hours. After a short centrifugation to bring down the water, the sample is transferred to a microcuvette and read at 820 mμ.

Step 4. Preparation of blanks and phosphorus standards: Two hundred microliters of 0.2 *N* potassium acetate in absolute ethanol is placed in a 2.0 ml. volumetric flask and brought to volume with absolute ethanol. An appropriate aliquot (60 to 120 μl) is placed in 12 separate 7 by 70 mm. tubes. The contents are completely dried in the centrifuge evaporator (60 to 90 minutes). Three tubes serve as the reagent blank. Two microliters of 0.6456, 1.2913, and 2.5826 mM. of phosphorus standard is pipetted to a group of 3 tubes, respectively, before adding 22.5 μl of 1:1 ashing mix. The blanks and standards are then carried through the rest of Step 3 simultaneously with the tissue sample.

Comments on Analytical Procedure.—

Step 1: It has been shown in other publications[6-8] that lipids are quantitatively coprecipitated with protein when TCA (5 to 10 per cent) is used. It has also been shown[6] that acid-soluble substances are significantly removed by the prescribed procedure. Furthermore, Robins and associates[8] have discussed and assumed that the various phospholipids are stable in the cold acid media (even plasmalogens).

If a normal C.S.F. contains 45 μg of inorganic phosphorus per 3 ml., in contrast to 0.75 μg of phospholipid phosphorus,[2] it can be seen that the inorganic phosphorus fraction could seriously contaminate the phospholipid fraction if it were not quantitatively removed. The efficiency of the present procedure in clearing the protein precipitate of inorganic phosphorus can be calculated, if it is assumed that 25 μl of solution remains after each aspiration. After the initial precipitation of protein and removal of the supernatant 0.45 μg of inorganic phosphorus remains. After the first wash only 0.02145 μg remains and after the second wash only 0.00105 μg of inorganic phosphorus remains. This is an insignificant contamination of 0.14 per cent of the phospholipid phosphorus fraction with inorganic phosphorus in normal C.S.F. Furthermore, if it is assumed that no inorganic phosphorus was adsorbed to the protein, then this calculation was borne out experimentally when no phosphorus could be detected either with or without digestion of the supernatant of the second TCA wash with the sensitive phosphorus procedure of Lowry and co-workers[3] (sensitivity of 0.003 μg of phosphorus).

Step 2: In another publication[6] the alcoholic extraction of phospholipids from the protein appeared quantititative (approximately 99 per cent).

Step 3: Lowry and associates[3] found the final optimal sulfuric acid concentration for color development is 0.9 N plus enough extra acid (0.1 N) to acidify the sodium acetate of the ascorbic acid-molybdic acid reagent. They stated that there is at least a 10 to 15 per cent tolerance in final acidity. The color developer must be kept in an ice bath and used within 1 hour. Each sample must be mixed immediately and thoroughly after the addition of the color developer.

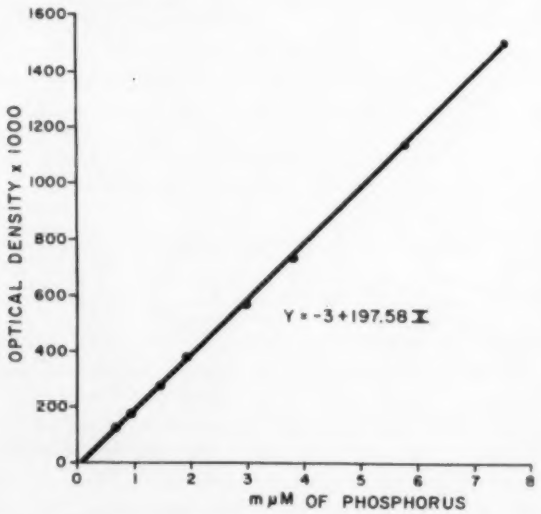

Fig. 1.—Phosphorus standard curve for the proposed procedure.

Step 4: In Fig. 1 is presented a phosphorus standard curve by the proposed procedure. By inspection, the line is straight from 0.7263 mμM (0.02 μg) to 7.5670 mμM (0.23 μg) of phosphorus. The equation of the line $Y = -3 + 197.58\ X$ was calculated by the analysis of least squares.[9]

Calculations.—From the equation of the line of the phosphorus standard curve, the mμM of phospholipid are calculated for the sample. If 50 μl of the 1 ml. lipid extract of 3.0 ml. of C.S.F. is used, then 1,100 times the mμM found in the sample is the mμM of total phospholipid per 100 ml. of C.S.F.

RESULTS AND DISCUSSION

The coefficient of variation[9] with the proposed procedure is 4.7 per cent (Table I). Therefore, if a number of extractions are carried out on a given C.S.F. specimen, it can be expected from the proposed procedure that 67 per cent of all the determinations will fall within ±4.7 per cent of the mean.

Experiments were designed to test the reproducibility of the proposed procedure on several different work days. C.S.F. specimens were stored at 4° C. between extractions. Two experiments gave the following results: analysis of C.S.F. A yielded 610, 556, and 570 mμM per 100 ml. of C.S.F., initially,

after 1 day, and after 6 days, respectively. Analysis of C.S.F. B yielded 427 and 406 mμM per 100 ml. of C.S.F., initially and after 1 day, respectively. With a coefficient of variation of 4.7 per cent, the data indicate that there is no significant difference between extractions. It can also be gleaned from this data that phospholipids in the C.S.F. do not deteriorate up to 6 days when stored at 4° C.

TABLE I. REPRODUCIBILITY OF THE PROPOSED PROCEDURE

DETERMINATION NO.	TOTAL PHOSPHOLIPIDS mμM/100 ML. OF C.S.F.
1	458
2	445
3	424
4	438
5	424
6	405
7	445
8	418
9	407
10	391
11	426
Mean	426
Standard deviation	±20
Coefficient variation	4.7%

The data in Table II illustrate on 4 different C.S.F. specimens the reproducibility of the proposed extraction procedure on different amounts of C.S.F. With a coefficient of variation of 4.7 per cent, the data indicate that there is no significant difference between extraction of 1.0, 2.0, or 3.0 ml. of C.S.F. It is interesting to note, however, that as a rule the less C.S.F. extracted, the higher the value. No good experimental explanation has been found. A fourth ethanol extract (60° or 98° C.); chloroform-methanol (2:1) substitution for ethanol; and increasing the organic solvent volume have all been employed without avail to correct this tendency.

TABLE II. REPRODUCIBILITY OF THE PROPOSED EXTRACTION PROCEDURE ON DIFFERENT AMOUNTS OF C.S.F.

CSF REFERENCE	μl OF C.S.F. USED			MEAN
	125	250	375	
C	805	839		822
D	625	605	586	605
E	464	416	402	427
F	631	579		605

All experiments were carried out on 120 μl of 1.0 ml. of lipid extract equivalent to 1.0, 2.0, or 3.0 ml. of C.S.F. unless otherwise specified; hence, each determination was done on 125, 250, or 375 μl of C.S.F., respectively. All phospholipid values are expressed in mμM per 100 ml. of C.S.F. and are the averages of triplicate determinations.

The extraction of lipids from a C.S.F. specimen by the Sperry-Brand procedure was compared to the proposed procedure. For the Sperry-Brand procedure 1 ml. of C.S.F. was extracted in triplicate. The lipid extract was purified by Sperry-Brand's procedure 2. After purification the solution was evaporated to dryness at 38° C. in a nitrogen stream without transferring. The residue obtained was dissolved in 1.5 ml. of chloroform-methanol (2:1). A

blank which contained 1 ml. of doubly distilled water in place of C.S.F. was carried through the Sperry-Brand procedure. Total phosphorus was determined as described in Step 3 except that 500 μl aliquots were dried. The agreement was good for these two different extraction procedures, 584 and 545 mμM 100 ml. of C.S.F. for the proposed method and the Sperry-Brand procedure, respectively.

SUMMARY

An analytic method is presented which is capable of determining 0.7 mμM (0.02 μg) of phospholipids with a coefficient of variation of about 5 per cent. It is based on the co-precipitation of proteins and lipids and the colorimetric determination of phosphorus on a suitable lipid extract of the protein. Several tests for the validity of the proposed procedure on C.S.F. are presented.

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A MEDIUM CONTAINING NUCLEIC ACID, MALTOSE, AND ANTIBIOTICS FOR THE ISOLATION OF GROUP A HEMOLYTIC STREPTOCOCCI

ROBERT J. ROANTREE, M.D., LOWELL A. RANTZ, M.D., AND
ELIZABETH HAINES, B.S.
SAN FRANCISCO, CALIF.

MEDIA recommended for the primary isolation of Group A hemolytic streptococci from respiratory sources that are low in dextrose content and contain sheep blood are satisfactory when employed by experienced persons. It seemed possible that adjuvants which would enhance the amount of hemolysis and interfere with the growth of certain organisms usually present in the nasopharyngeal flora might increase the ease with which less skilled persons could isolate these important organisms from clinical materials.

Previous investigators[1, 2] have described augmentation of production of streptolysin S, the active substance causing hemolysis on blood agar, by nucleic acid and maltose. Other studies have demonstrated the resistance of group A streptococci to the antibacterial effects of neomycin and polymyxin.[3] This paper described the incorporation of these substances into a medium which simplifies the isolation of group A streptococci.

METHODS

Basal Medium.—The basal medium used in nearly all of the studies to be described was beef heart infusion agar (Difco). It was employed in a concentration of 40.0 Gm. per liter, and was usually dissolved in a solution of nucleic acid in distilled water which had been adjusted to a pH of 7.0. Sterilization was accomplished by autoclaving at 15 pounds of pressure for 15 minutes.

In certain studies other commercially available dehydrated media were employed. Each was used in the concentration recommended by the manufacturer except that it was dissolved in a solution of nucleic acid.

Nucleic Acid.—Various commercially available nucleic acids were investigated. Each was dissolved in distilled water in appropriate concentrations and the solution was neutralized by the addition of 4.0 per cent sodium hydroxide solution. In certain experiments the more concentrated solutions were diluted with distilled water after neutralization and before addition of the basal medium.

Maltose.—Maltose solutions of appropriate concentration were prepared and sterilized by Seitz filtration and added aseptically to the medium when the finished plates were prepared.

Antibiotics.—Neomycin and polymyxin were obtained as sterile powders in vials for parenteral administration, dissolved in sterile normal salt solution, appropriately diluted, and stored in the Deepfreeze at -20° C. until used.

From the Department of Medicine, Stanford University School of Medicine, San Francisco, Calif.

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Blood.—Preliminary studies not reported demonstrated that the optimum defibrinated concentration of sheep blood to be used in the augmented medium was 5 per cent and the amount was employed throughout the study.

Group A Hemolytic Streptococci.—Forty-nine strains of group A hemolytic streptococci isolated from clinical sources were used throughout the study. They were maintained by regular transfer in blood broth and were stored in the Deepfreeze when interruptions occurred in the work. Serologic typing was not done.

In all experiments the size of the hemolytic zone was estimated by measuring the diameter around several well-isolated colonies on each plate and recording the median of these values as a point on a chart such as that represented in Fig. 1. The absolute values varied from experiment to experiment but there was considerable consistency in the relative ability of the various strains to cause hemolysis on blood agar plates.

A control consisting of heart infusion agar containing 5.0 per cent sheep blood only was included in each experiment. The more significant studies were repeated, often on several occasions.

Clinical Trial.—A clinical trial of the augmented medium was carried out employing throat swabs obtained from children with respiratory infections attending the Pediatric Outpatient Clinic of Stanford University Hospitals. Each swab was vigorously agitated in 1.0 ml. of broth and 2 loopfuls of the resulting suspension of bacteria were plated on the augmented medium and a similar amount on heart infusion agar containing 5 per cent sheep blood but no adjuvants.

RESULTS

Nucleic Acid.—As the concentrations of nucleic acid were increased from 0 to 0.9 per cent, there was an enhancement of hemolysis through the 0.6 per cent level after which a sharp decline occurred. Median hemolytic zone size at the optimum concentration was 20 per cent greater at both 24 and 48 hours in the augmented, as compared with the control, medium. An increased clarity of the hemolytic zone was very evident and of as great significance as the increased size of the cleared area.

Five commercially available nucleic acid preparations were studied in parallel at the 0.6 per cent level. Two, manufactured by Schwarz Laboratories and Bios Laboratories, Inc., were distinctly inferior to 3 others prepared by the Nutritional Biochemical Corporation, the California Foundation for Biochemical Research, and the Roussel Laboratories* which were comparable. The latter was used in all of the subsequent studies.

Maltose.—Preliminary study of the basal media containing only maltose as an adjuvant demonstrated enhancement of hemolysis. Detailed investigation revealed that the optimum concentration in a medium containing 6.0 Gm. of nucleic acid per liter was between 0.04 and 0.10 Gm. per cent. The amount used in subsequent work was 0.05 Gm. per cent.

The increments in zone size obtained when 36 strains of group A streptococci were grown on the augmented medium containing 6.0 Gm. of nucleic acid and 0.5 Gm. of maltose per liter, and on the control medium are depicted in the first half of Fig. 1. After 24 hours of incubation there was an approximately

*Address: 155 East 44th Street, New York 17, N. Y.

50 per cent, and after 48 hours a 100 per cent, increment in median zone diameter. It is of interest that a few strains seemed unable to respond to the stimulation of the adjuvants.*

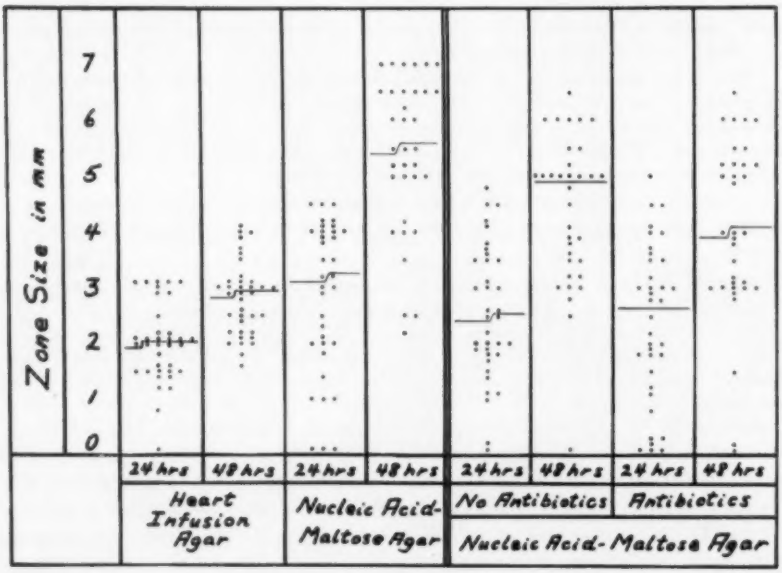

Fig. 1.—Effect on hemolytic zone size of addition of nucleic acid and maltose to heart infusion agar (left hand panel), and of polymyxin and neomycin to nucleic acid maltose agar (right hand panel).

Blood Agar Base.—A number of commercially available, dehydrated blood agar bases were studied to determine their suitability for the isolation of group A streptococci after augmentation with nucleic acid and maltose. Median zone diameter was greatest on heart infusion agar† and brain veal agar† and slightly smaller on veal infusion agar.† The B.B.L. blood agar base‡ and Difco blood agar base† were distinctly inferior. Two different lots of trypticase soy agar‡ completely failed to support hemolysin production by two-thirds of the test strains when nucleic acid and maltose were added in spite of the fact that the unaltered medium permitted the development of excellent hemolysis comparable to that of the other media being tested.

Addition of Antibiotics.—Previous work had demonstrated that group A streptococci are resistant to the antibacterial action of neomycin. This antibiotic was incorporated in various concentrations in blood agar plates prepared from the basal medium and 42 strains of the test group of hemolytic streptococci

*Continuing study has revealed a few strains of Group A streptococci whose hemolysin production is substantially reduced on the augmented medium without antibiotics.

†Difco Laboratories, Detroit, Mich.

‡Baltimore Biological Laboratory, Baltimore, Md.

were studied. Every one grew well in the presence of 50 μg per milliliter although 13 exhibited reduced or absent hemolysis at this level of antibiotic. All were actively hemolytic in the presence of 12.5 μg per milliliter of neomycin.

A similar study employing polymyxin revealed no inhibition of growth or hemolysis formation in the presence of 25 μg per milliliter, the highest level tested.

After clinical trial it was determined that the most satisfactory concentrations of antibiotics for the inhibition of unwanted organisms in the nasopharyngeal flora were 2.0 μg of neomycin and 10.0 μg of polymyxin per milliliter.

The ability of the nucleic acid-maltose medium with and without these amounts of antibiotic to support hemolysis was studied. The results of a typical experiment employing 33 of the test strains of group A streptococci are portrayed in the second half of Fig. 1. There was no reduction in median zone diameter after 24 hours of incubation; however, a few strains had grown but had not produced good hemolysis. This effect persisted but was less marked after 48 hours of incubation. At this time there was a 20 per cent decrement in median zone diameter in the presence of the antibiotics.

Clinical Trial.—A clinical trial of the augmented medium containing 6.0 Gm. of nucleic acid and 0.5 Gm. of maltose per liter was carried out. At this time all of the studies described above had not been completed, and 5.0 μg of neomycin and 10.0 μg of polymyxin per milliliter were added. Later it was determined that the desirable inhibitory effect on the respiratory flora could be obtained with 2.0 μg per milliliter of neomycin. It may be assumed that the recovery of group A streptococci under these circumstances would be at least as satisfactory as when the larger amount was employed.

The basal and the augmented media, each containing 5.0 per cent sheep blood, were compared in the study of 755 consecutive throat cultures. Sixty-six strains of group A hemolytic streptococci were recovered. The number of colonies of these organisms were approximated. When there were less than 50 colonies on the control plate (37 cultures), the augmented medium failed to recover the organism on 8 occasions. When 20 per cent or more of the colonies on the control plate were group A streptococci, there was almost complete uniformity between the two preparations. In only 2 of 29 instances of this type was the number of organisms on the augmented plate sufficiently reduced (less than 50 colonies) so that a diagnosis of streptococcal disease might have been overlooked.

Thirty strains of hemolytic streptococcus of groups C and G were also isolated. These organisms grew and produced hemolysis well on the augmented medium.

More recent, but not so carefully controlled, information indicates that the same medium containing 2.0 μg per milliliter of neomycin is entirely comparable to the basal medium in its ability to recover group A streptococci.

The principal advantages of this medium lay in the simplicity of its application and the precision with which hemolytic streptococci could be recognized. Beta hemolysis was brisk and sharply defined. Growth of alpha hemolytic

streptococci and pneumococci invariably resulted in the production of large, vivid green zones without the slightest trace of hemolysis which is such a constant source of confusion to the inexperienced. The growth of nearly all micrococci and influenza bacilli was completely inhibited, and the number of Neisseria was strikingly reduced so that the plates revealed nearly pure growths of various streptococci and pneumococci. Reading of cultures after 24 hours of incubation could be accomplished readily by simply holding them to a light. Because overgrowth of other organisms was prevented, an additional 24-hour period of incubation was quite feasible and provided an additional check in doubtful instances.

The medium was further tested in the field by supplying it over a period of several months to a small group of pediatricians in the Palo Alto area, who inoculated, incubated, and read plates prepared from swabs obtained from their own patients, following which the preparations were rescored by one of the authors. No difficulties were encountered and these physicians, with little experience in diagnostic microbiology, were able to interpret the results of their cultures accurately from the beginning. These practitioners were so enthusiastic about the results obtained that it became necessary for a commercial laboratory to undertake the manufacture of the medium for distribution in the area after the study was terminated.

SUMMARY

This report describes the development of an augmented medium for the primary isolation of group A hemolytic streptococci from the respiratory tract of human beings. The addition of 6.0 Gm. per liter of yeast nucleic acid and 0.5 Gm. of maltose increases the size and enhances the clarity and sharpness of the hemolytic zone produced by these organisms on sheep blood agar.

In a limited study, certain nucleic acids and blood agar bases were superior.

The introduction of 2.0 μg per milliliter of neomycin and 10.0 μg per milliliter of polymyxin into the nucleic acid and maltose medium did not significantly interfere with the growth of, or hemolysis production by, group A streptococci but did materially enhance the ease of isolation of these organisms by inhibiting the growth of Micrococcus, Hemophilus, and Neisseria.

A controlled clinical trial in the hospital clinic demonstrated the suitability of the medium for isolation of group A streptococci from throat swabs. A field trial by a group of physicians without previous special training in microbiologic techniques demonstrated its practical value.

It is believed that commercial availability of this medium in plastic, disposable Petri dishes would permit the rapid and precise diagnosis of hemolytic streptococcal respiratory infection by smaller laboratories and by physicians in their offices.

REFERENCES

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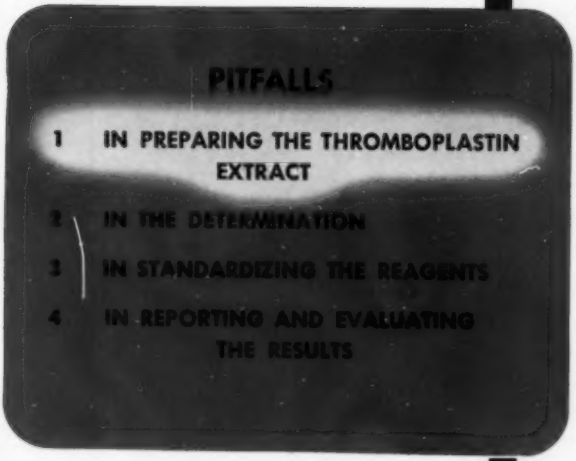
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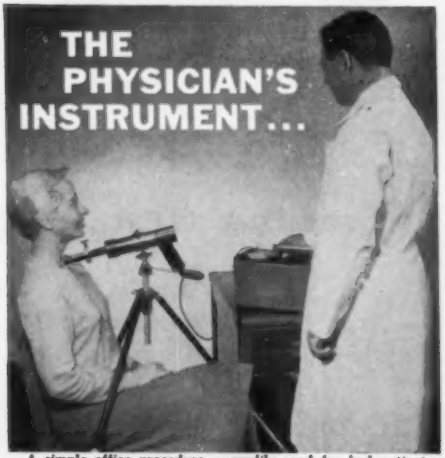

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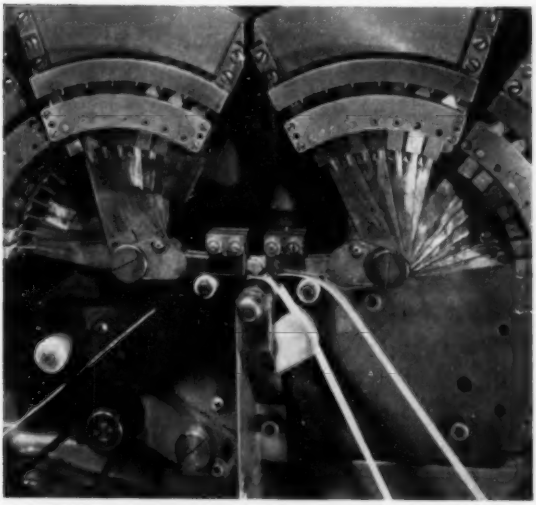

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E.
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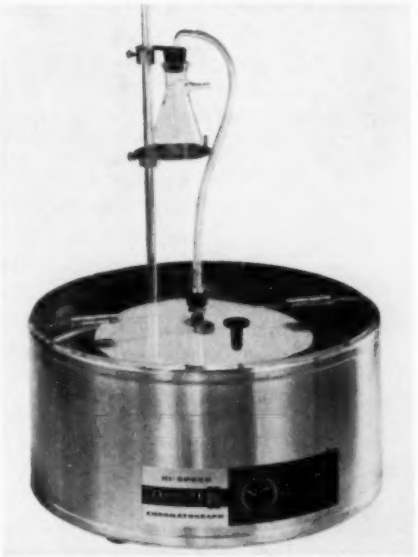

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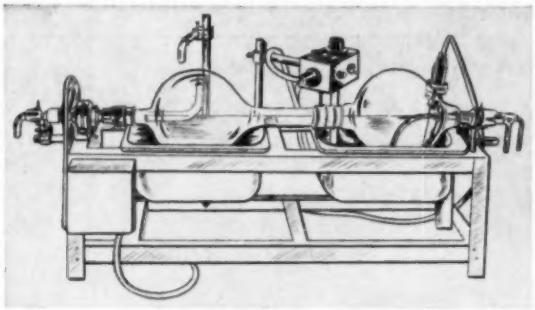

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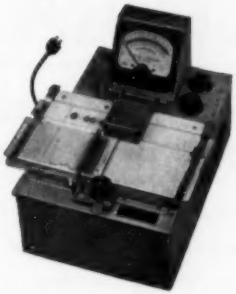

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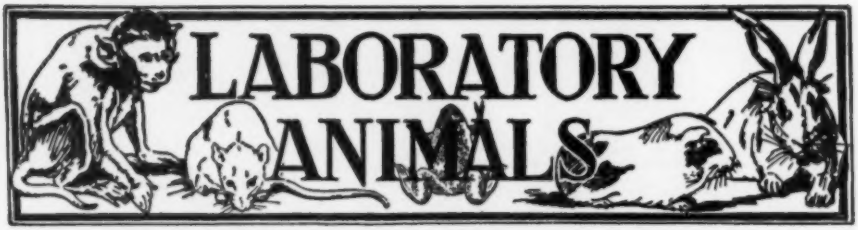

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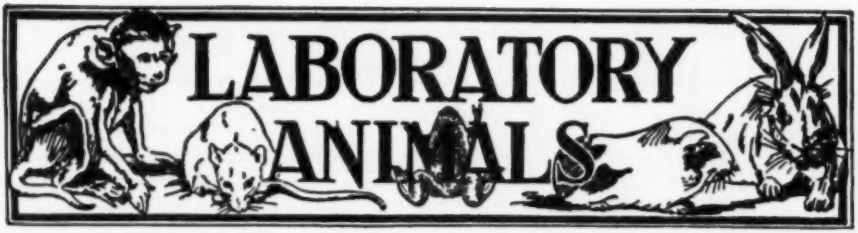

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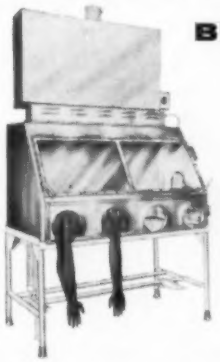

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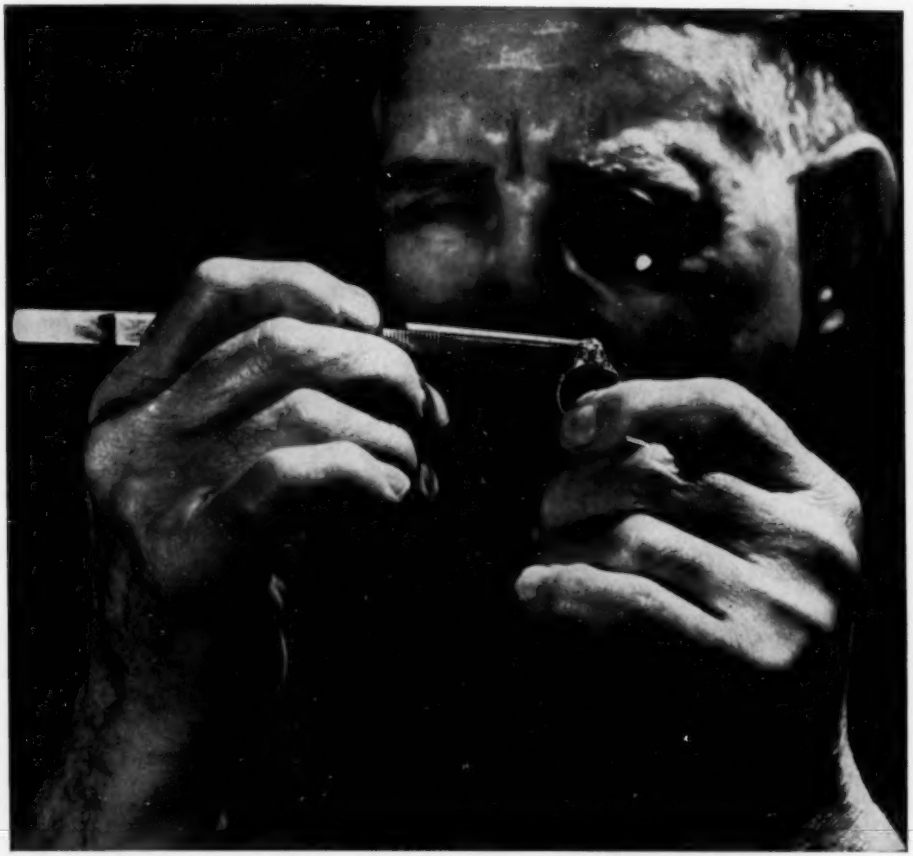

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